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(54) Title: NEW FORMULATION OF ANTI VISTA ANTIBODY

(57) Abstract: The present invention provides an anti-VISTA antibody which is suitable for pharmaceutical development, as pharmaceutical compositions comprising this antibody, and methods of treating VISTA-mediated diseases.

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NEW FORMULATION OF ANTI VISTA ANTIBODY

INTRODUCTION

Development of a therapeutic antibody is a complex process. Various physiochemical and functional liabilities can compromise the production or the therapeutic efficacy of antibodies. The present disclosure provides an anti-VISTA antibody which is suitable for pharmaceutical development, as pharmaceutical compositions comprising this antibody, and methods of treating VISTA-mediated diseases.

Immunotherapy has been a game-changer in the field of cancer therapy. In order to ensure that an immune inflammatory response is not constantly activated once tumour antigens have stimulated a response, multiple controls or "checkpoints" are in place or activated. VISTA (V-Domain Ig Suppressor of T Cell Activation) is a negative checkpoint control protein that regulates T cell activation and immune responses. VISAT is a member of the B7 family which comprises several immune checkpoint proteins such as PD-L1. However, unlike of the members of this family, VISTA comprises a single unusually large Ig-like V-type domain. In addition, VISTA cytoplasmic tail domain contains several docketing sites for effector proteins, suggesting that VISTA could potentially function as both a receptor and a ligand.

Physiologically, VISTA exerts a regulatory function on the immune system at several levels, particularly by modulating T cells activation. More recently, VISTA was identified as the earliest checkpoint regulator of peripheral T cell tolerance, particularly in the maintenance of naïve T cell quiescence. In the context of cancer, VISTA is upregulated on immunosuppressive tumour infiltrating leukocytes such as inhibitory regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). The presence of VISTA in the tumour microenvironment hinders effective T cell responses and has been implicated in a number of human cancers including prostate, colon, skin, pancreatic, and lung.

Several antagonistic anti-VISTA antibodies have been described which can be used for the treatment of cancer (ElTanbouly et al. *Clin Exp Immunol*. 200(2):120-130 (2020); Mehta et al. *Sci Rep*. **10**(1):1 5171 (2020); Yuan et al. *Trends Immunol*. **42**(3): 209-227 (2021); Tagliamento et al. *Immunotargets Ther*. **10**: 185-200 (2021); Thakkar et al. *J Immunother Cancer*. **10**(2): e003382 (2022); WO 2015/097536;

WO 2016/094837; WO 2017/181139; WO 2019/183040). In particular, WO 2016/094837 discloses an antibody capable of inhibiting VISTA suppression of the anti-tumour immune response, thereby conferring protective anti-tumour immunity. However, this antibody comprises several potential Asn residues potentially susceptible to deamidation which could thus affect drug efficacy and clinical and manufacturing development.

Moreover, antibody drugs are unstable due to their large molecular weight, complex structure, being susceptible to degradation, being polymerised, or undesired chemical modification. Studies on stable formulations of antibody drugs are particularly important in order to make antibodies suitable for administration, and to maintain stability during the storage and the subsequent use.

Thus there is a need to develop pharmaceutical compositions (formulations) comprising a homogenous, safe and efficacious anti-VISTA antibody, which are suitable for administration.

SUMMARY

The following aspects are provided herein:

In a first aspect the present disclosure provides a pharmaceutical composition comprising an isolated antibody, or antigen-binding fragment thereof, which specifically binds VISTA; and a pharmaceutically acceptable carrier and/or excipient. This antibody has the heavy chain and light chains provided herein, *e.g.*, a heavy chain of sequence represented by SEQ ID NO:21 and a light chain of sequence represented by SEQ ID NO:22. In particular, the present anti-VISTA antibody has an aspartic acid at position 55. The anti-VISTA antibody disclosed herein is preferably not susceptible to deamidation.

Preferably, the antibody is a monoclonal antibody, more preferably a humanised antibody.

In an instance, the concentration of the monoclonal anti-VISTA antibody is comprised between 5 and 100 mg/mL, preferably between 10 and 70 mg/mL, more preferably between 15 and 50 mg/mL, most preferably between 20 and 30 mg/mL. in a preferred instance, the concentration of the monoclonal anti-VISTA antibody is 20 mg/mL.

Ina another aspect, the present disclosure provides a pharmaceutical composition comprising the anti-VISTA antibody and a buffering agent, preferably a citrate buffer, a phosphate buffer, or a histidine buffer, more preferably a histidine buffer.

In an instance, the buffering agent is a histidine buffer at a concentration comprised between 5 and 45 mM, preferably 10 and 40 mM, more preferably 15 and 35 mM, most preferably 20 and 30 mM. In a preferred instance, the buffering agent is a histidine buffer at a concentration of 25 mM.

In another aspect, the present disclosure provides a pharmaceutical composition comprising the anti-VISTA antibody and a tonicity modifier. Preferably, the tonicity modifier is selected in the group consisting of polyhydric sugar alcohols, for example trihydric or higher sugar alcohols, such as glycerine, erythritol, arabitol, xylitol, sorbitol and mannitol, salts and amino acids; more preferably, a salt selected in the group consisting of sodium chloride, sodium succinate, sodium sulphate, potassium chloride, magnesium chloride, magnesium sulphate, and calcium chloride; even more preferably NaCl, $MgCl_2$, and/or $CaCl_2$, most preferably NaCl.

In an instance, the tonicity modifier is present in the formulation at a concentration comprised between 0 mM and 300 mM, preferably between 50 mM and 250 mM, more preferably between 100 mM and 200 mM.

In an instance, the tonicity modifier is NaCl at a concentration comprised between 0 mM and 300 mM, preferably between 50 mM and 250 mM, more preferably between 100 mM and 200 mM NaCl. In a preferred instance, the pharmaceutical composition comprises NaCl at a concentration of 150 mM NaCl.

In another aspect, the present disclosure provides a pharmaceutical composition comprising a non-ionic surfactant, preferably a polysorbate, *e.g.*, Polysorbate 20 or Polysorbate 80.

In an instance, the non-ionic surfactant is Polysorbate 80 at a concentration between about 0.001% and about 0.5% (v/v), between about 0.002% and about 0.2% (v/v), between about 0.003% and about 0.1% (v/v), between about 0.004% and about 0.009% (v/v), between about 0.005% and about 0.008% (v/v), or between about 0.006% and about 0.007%. (v/v). In a preferred instance, the pharmaceutical composition comprises Polysorbate 80 at a concentration of 0.006% (v/v).

In another aspect, the present disclosure provides a pharmaceutical composition having a pH comprised between about 3.0 and about 9.0, between about 4.0 and about 8.0, between about 5.0 and about 8.0, between about 5.0 and about 7.0, between about 5.0 and about 6.5, between about 5.5 and about 8.0, between about 5.5 and about 7.0, or between about 5.5 and about 6.5. In an instance, the pH of the pharmaceutical composition is about 6.5.

Preferably, the pharmaceutical composition comprises 25 mM Histidine, 150 mM NaCl, 0.006% Polysorbate 80 (v/v), pH 6.5, in addition to the monoclonal anti-VISTA antibody disclosed herein.

In another aspect, the monoclonal anti-VISTA antibody or the immunoconjugate or the pharmaceutical composition disclosed herein are for use in the treatment of a VISTA-mediated disease, notably a cancer, in a patient. Preferably, this use comprises inducing an immune response in the patient. Preferably, the immune response includes induction of CD4+ T cell proliferation, induction of CD8+ T cell proliferation, induction of CD4+ T cell cytokine production, and induction of CD8+ T cell cytokine production. Preferably, the use disclosed herein comprises activation of the effector functions of the antibody.

In a preferred aspect, the therapeutic use disclosed herein comprises the administration of a second therapeutic agent. This second therapeutic agent is advantageously an anti-PD-1 antibody or an anti-PD-L1 antibody.

Preferably, the cancer is selected from bladder cancer, breast cancer, cervical cancer, colon cancer, endometrial cancer, oesophageal cancer, fallopian tube cancer, gall bladder cancer, gastrointestinal cancer, head-and-neck cancer, haematological cancer (e.g., leukaemia, lymphoma, or myeloma), laryngeal cancer, liver cancer, lung cancer, lymphoma, melanoma, mesothelioma, ovarian cancer, primary peritoneal cancer, salivary gland cancer, sarcoma, stomach cancer, thyroid cancer, pancreatic cancer, renal cell carcinoma, glioblastoma, and prostate cancer.

FIGURE LEGENDS

Figure 1: Charge variants identified with cation exchange chromatography, pH gradient. *Left panel*: Several peaks are visible for product 1, comprising Ab3, showing that it is subject to degradation, specifically deamidation at amino acid residue 55.

Right panel: A singly peak is visible for product 2, comprising Ab1, showing that it is no longer susceptible to deamidation at amino acid residue 55, and is stable.

Figure 2: Graphic representation of averaged data obtained with antibodies of the third series in the three experiments (N=3) in direct rhVISTA ELISA. Filed square: Ab1, diamond: Ab3 batch 1, inverted triangle: Ab3 batch 2, triangle: IgG1 anti-VISTA (positive control), open circles: c9G4 (negative control), open circles and dotted line: anti-hVISTA polyclonal antibody (positive control).

Figure 3: Graphic representation of averaged data obtained with antibodies of the third series in the three experiments (N=3) in indirect rhVISTA ELISA. Filed square: Ab1, diamond: Ab3 batch 1, inverted triangle: Ab3 batch 2, triangle: IgG1 anti-VISTA (positive control), open circles: c9G4 (negative control), open circles and dotted line: anti-hVISTA polyclonal antibody (positive control).

Figure 4: Evaluation of T cells activation and cytokines release in CHO-VISTA coculture with PBMC: schematic representation of the experiment.

Figure 5: Evaluation of T cells activation and cytokines release in CHO-VISTA coculture with PBMC: Anti-VISTA Ab1 competent induced T cells activation and cytokines release in CHO-VISTA coculture with PBMC. (Donor 119). Ab1 silent: Ab1 variant with the N298A mutation.

Figure 6: Binding of rhVISTA-Fc and rhVISTA-TagHis on rhVSIG3-Fc =f([anti-VISTA Ab1]).

Figure 7: In vivo activity of the competent anti-VISTA Ab1 in a MC38 xenograft model.

Figure 8: In vivo activity of the silent anti-VISTA Ab1 (N298A variant) in a MC38 xenograft model.

Figure 9: Types of derivative plots and melting temperatures (Tm) obtained at T0 step 1 for all the conditions.

Figure 10: MODDE analysis of TSA results at T0 of step 1. The values in the squares indicate the weight of the factor when it is significant (Interval does not cross the zero).

Figure 11: Analysis of remaining mAb 1 in step 1. The values in the squares indicate the weight of the factor when it is significant (Interval does not cross the zero).

Figure 12: Figure 4: Analysis of monomers content in step 1. The values in the squares indicate the weight of the factor when it is significant (Interval does not cross the zero).

DETAILED DESCRIPTION

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. The practice of the invention employs, unless other otherwise indicated, conventional techniques or protein chemistry, molecular virology, microbiology, recombinant DNA technology, and pharmacology, which are within the skill of the art. Such techniques are explained fully in the literature (see *e.g.*, Ausubel *et al.*, Short Protocols in Molecular Biology, Current Protocols; 5th Ed., 2002; Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1985; and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press; 3rd Ed., 2001). The nomenclatures used in connection with, and the laboratory procedures and techniques of, molecular and cellular biology, protein biochemistry, enzymology and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

The present invention will become more fully understood from the detailed description given herein and from the accompanying drawings, which are given by way of illustration only and do not limit the intended scope of the invention.

Definitions

Unless specifically defined, all technical and scientific terms used herein have the same meaning as commonly understood by a skilled artisan in chemistry, biochemistry, cellular biology, molecular biology, and medical sciences.

The term **"about"** or **"approximately"** refers to the normal range of error for a given value or range known to the person of skills in the art. It usually means within 20%, such as within 10%, or within 5% (or 1% or less) of a given value or range.

As used herein, the expression "**Antibody-dependent cell-mediated cytotoxicity**", **"Antibody-dependent cellular cytotoxicity"** or "**ADCC**" refers to a form of cytotoxicity in which an immunoglobulin bound onto Fc receptors (FcRs) present on certain cytotoxic effector cells enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. Lysis of the target cell is extracellular, requires direct cell-to-cell contact, and does not involve complement. Cell destruction can occur, for example, by lysis or phagocytosis. To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Patent Nos. 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).

"Antibody-dependent phagocytosis" or **"ADCP"** or **"opsonisation"** as used herein refers to the cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognise bound antibody on a target cell and subsequently cause phagocytosis of the target cell.

As used herein, **"administer"** or **"administration"** refers to the act of injecting or otherwise physically delivering a substance as it exists outside the body (*e.g.*, an anti-VISTA antibody provided herein) into a patient, such as by mucosal, intradermal, intravenous, intramuscular delivery and/or any other method of physical delivery described herein or known in the art. When a disease, or a symptom thereof, is being treated, administration of the substance typically occurs after the onset of the disease or symptoms thereof. When a disease, or symptoms thereof, are being prevented, administration of the substance typically occurs before the onset of the disease or symptoms thereof.

As used herein, an **"antagonist"** or **"inhibitor"** refers to a molecule that is capable of inhibiting or otherwise decreasing one or more of the biological activities of a target protein, such as VISTA. In some embodiments, an antagonist of VISTA (*e.g.*, an

antagonistic antibody provided herein) can, for example, act by inhibiting or otherwise decreasing the activation and/or cell signalling pathways of the cell expressing VISTA (*e.g.*, a VISTA-bearing tumour cell, a regulatory T cell, a myeloid-derived suppressor cell or a suppressive dendritic cell), thereby inhibiting a biological activity of the cell relative to the biological activity in the absence of the antagonist. For example, an antagonist of VISTA may inhibit VISTA's suppressive effects on T cell immunity (CD4+ and/or CD8+ T cell immunity) and/or the expression of proinflammatory cytokines. More specifically, an antagonist of VISTA may block or decrease the interaction of VISTA with at least one of its ligands, including VSIG3, PSG-L1, VSIG8, and LRIG1. Even more specifically, an antagonist of VISTA may block or decrease the interaction of VISTA with either of VSIG3 or PSG-L1. Preferably, an antagonist of VISTA may block or decrease the interaction of VISTA with PSG-L1 at acidic pH (*i.e.*, at pH between 5.9 and 6.5). In some embodiments the antibodies provided herein are antagonistic anti-VISTA-1 antibodies. Certain antagonistic antibodies substantially or completely inhibit one or more of the biological activities of said antigen. For example, an antagonistic anti-VISTA antibody may inhibit VISTA's suppressive effects on T cell immunity (CD4+ and/or CD8+ T cell immunity) and/or the expression of proinflammatory cytokines. More specifically, an antagonistic anti-VISTA antibody may block or decrease the interaction of VISTA with at least one of its ligands, including VSIG3, PSG-L1, VSIG8, and LRIG1. Even more specifically, an antagonistic anti-VISTA antibody may block or decrease the interaction of VISTA with either of VSIG3 or PSG-L1. Preferably, an antagonistic anti-VISTA antibody may block or decrease the interaction of VISTA with PSG-L1 at acidic pH (*i.e.*, at pH between 5.9 and 6.5).

The terms **"antibody"** and **"immunoglobulin"** or **"Ig"** are used interchangeably herein. These terms are used herein in the broadest sense and specifically cover monoclonal antibodies (including full length monoclonal antibodies) of any isotype such as IgG, IgM, IgA, IgD, and IgE, polyclonal antibodies, multispecific antibodies, chimeric antibodies, and antibody fragments, provided that said fragments retain the desired biological function. These terms are intended to include a polypeptide product of B cells within the immunoglobulin class of polypeptides that is capable of binding to a specific molecular antigen and is composed of two identical pairs of polypeptide chains inter-connected by disulfide bonds, wherein each pair has one heavy chain (about 50-70 kDa) and one light chain (about 25 kDa) and each amino-terminal portion of each chain includes a variable region of about 100 to about 130 or more amino acids and

each carboxy-terminal portion of each chain includes a constant region (*See*, Borrebaeck (ed.) (1995) Antibody Engineering, Second Ed., Oxford University Press.; Kuby (1997) Immunology, Third Ed., W.H. Freeman and Company, New York). Each variable region of each heavy and light chain is composed of three complementarity-determining regions (CDRs), which are also known as hypervariable regions and four frameworks (FRs), the more highly conserved portions of variable domains, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system. In some embodiments, the specific molecular antigen can be bound by an antibody provided herein includes the target VISTA polypeptide, fragment or epitope. An antibody reactive with a specific antigen can be generated by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors, or by immunising an animal with the antigen or an antigen-encoding nucleic acid.

Antibodies also include, but are not limited to, synthetic antibodies, monoclonal antibodies, recombinantly produced antibodies, multispecific antibodies (including bi-specific antibodies), human antibodies, humanised antibodies, camelised antibodies, chimeric antibodies, intrabodies, anti-idiotypic (anti-Id) antibodies, and functional fragments of any of the above, which refers a portion of an antibody heavy or light chain polypeptide that retains some or all of the biological function of the antibody from which the fragment was derived. The antibodies provided herein can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), any class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or any subclass (*e.g.*, IgG2a and IgG2b) of immunoglobulin molecule.

The terms **"anti-VISTA antibodies" "antibodies that bind to VISTA," "antibodies that bind to a VISTA epitope,"** and analogous terms are used interchangeably herein and refer to antibodies that bind to a VISTA polypeptide, such as a VISTA antigen or epitope. Such antibodies include polyclonal and monoclonal antibodies, including chimeric, humanised, and human antibodies. An antibody that binds to a VISTA antigen may be cross-reactive with related antigens. In some embodiments, an antibody that binds to VISTA does not cross-react with other antigens

such as *e.g.*, other peptides or polypeptides belonging to the B7 superfamily. An antibody that binds to VISTA can be identified, for example, by immunoassays, BIAcore, or other techniques known to those of skill in the art. An antibody binds to VISTA, for example, when it binds to VISTA with higher affinity than to any cross-reactive antigen as determined using experimental techniques, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISAs), for example, an antibody that specifically binds to VISTA. Typically, a specific or selective reaction will be at least twice background signal or noise and may be more than 10 times background. See, *e.g.*, Paul, ed., 1989, Fundamental Immunology Second Edition, Raven Press, New York at pages 332-336 for a discussion regarding antibody specificity. In some embodiments, an antibody "which binds" an antigen of interest is one that binds the antigen with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting a cell or tissue expressing the antigen, and does not significantly cross-react with other proteins. In such embodiments, the extent of binding of the antibody to a "non-target" protein will be less than about 10% of the binding of the antibody to its particular target protein as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIPA). With regard to the binding of an antibody to a target molecule, the term **"specific binding"** or **"specifically binds to"** or is **"specific for"** a particular polypeptide or an epitope on a particular polypeptide target means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labelled target. In this case, specific binding is indicated if the binding of the labelled target to a probe is competitively inhibited by excess unlabelled target. The term **"specific binding"** or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a K_D for the target of at least about 10^{-4} M, alternatively at least about 10^{-5} M, alternatively at least about 10^{-6} M, alternatively at least about 10^{-7} M, alternatively at least about 10^{-8} M, alternatively at least about 10^{-9} M, alternatively at least about 10^{-10} M, alternatively at least about 10^{-11} M, alternatively at least about 10^{-12} M, or greater. In some embodiments, the term "specific binding" refers to binding where a molecule binds to a particular polypeptide or epitope on a particular

polypeptide without substantially binding to any other polypeptide or polypeptide epitope. In some embodiments, an antibody that binds to VISTA has a dissociation constant (K_D) of ≤ 1μM, ≤ 100 nM, ≤ 10 nM, ≤ 1nM, or ≤ 0.1nM.

An "**antigen**" is a predetermined antigen to which an antibody can selectively bind. The target antigen may be a polypeptide, carbohydrate, nucleic acid, lipid, hapten or other naturally occurring or synthetic compound. In some embodiments, the target antigen is a polypeptide, including, for example, a VISTA polypeptide.

The term "**antigen binding fragment**," "**antigen binding domain**," "**antigen binding region**," and similar terms refer to that portion of an antibody which comprises the amino acid residues that interact with an antigen and confer on the binding agent its specificity and affinity for the antigen (*e.g.*, the complementarity determining regions (CDRs)).

The term "**antigen binding fragment,**" "**antigen binding domain,**" "**antigen binding region,**" and similar terms refer to that portion of an antibody which comprises the amino acid residues that interact with an antigen and confer on the binding agent its specificity and affinity for the antigen (*e.g.*, the complementarity determining regions (CDRs)). By the expression "**antigen-binding fragment**" of an antibody, it is intended to indicate any peptide, polypeptide, or protein retaining the ability to bind to the target (also generally referred to as antigen) of the said antibody, generally the same epitope, and comprising an amino acid sequence of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino residues, at least 70 contiguous amino acid residues, at least 80 contiguous amino acid residues, at least 90 contiguous amino acid residues, at least 100 contiguous amino acid residues, at least 125 contiguous amino acid residues, at least 150 contiguous amino acid residues, at least 175 contiguous amino acid residues, or at least 200 contiguous amino acid residues, of the amino acid sequence of the antibody. In a particular embodiment, the said antigen-binding fragment comprises at least one CDR of the antibody from which it is derived. Still in a preferred embodiment, the said antigen binding fragment comprises 2, 3, 4 or 5 CDRs, more preferably the 6 CDRs of the antibody from which it is derived.

The **"antigen-binding fragments"** can be selected, without limitation, in the group consisting of Fab, Fab', $(Fab')_2$, Fv, scFv (sc for single chain), Bis-scFv, scFv-Fc fragments, Fab2, Fab3, minibodies, diabodies, triabodies, tetrabodies, and nanobodies, and fusion proteins with disordered peptides such as XTEN (extended recombinant polypeptide) or PAS motifs, and any fragment of which the half-life time would be increased by chemical modification, such as the addition of poly(alkylene) glycol such as poly(ethylene) glycol ("PEGylation") (pegylated fragments called Fv-PEG, scFv-PEG, Fab-PEG, $F(ab')_2$-PEG or Fab'-PEG) ("PEG" for Poly(Ethylene) Glycol), or by incorporation in a liposome, said fragments having at least one of the characteristic CDRs of the antibody according to the invention. Among the antibody fragments, Fab has a structure including variable regions of light chain and heavy chain, a constant region of a light chain, and the first constant region of a heavy chain (CH1), and it has one antigen binding site. Fab' is different from Fab in that it has a hinge region including one or more cysteine residues at C terminus of heavy chain CH1 domain. F(ab')2 antibody is generated as the cysteine residues of the hinge region of Fab' form a disulfide bond. Fv is a minimum antibody fragment which has only a heavy chain variable region and a light chain variable region, and a recombination technique for producing the Fv fragment is described in International Publication WO 88/10649 or the like. In double chain Fv (dsFv), the heavy chain variable region and light chain variable region are linked to each other via a disulfide bond, and, in single chain Fv (scFv), the heavy chain variable region and light chain variable region are covalently linked to each other via a peptide linker in general. Those antibody fragments can be obtained by using a proteinase (*e.g.*, Fab can be obtained by restriction digestion of whole antibody with papain, and F(ab')2 fragment can be obtained by restriction digestion with pepsin), and it can be preferably produced by genetic engineering techniques. Preferably, said "antigen-binding fragments" will be constituted or will comprise a partial sequence of the heavy or light variable chain of the antibody from which they are derived, said partial sequence being sufficient to retain the same specificity of binding as the antibody from which it is descended and a sufficient affinity, preferably at least equal to 1/100, in a more preferred manner to at least 1/10, of the affinity of the antibody from which it is descended, with respect to the target. Such antibody fragments can be found described in, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989); Myers (ed.), Molec. Biology and Biotechnology: A Comprehensive Desk Reference, New York: VCH Publisher, Inc.; Huston *et al.*, *Cell Biophysics*, 22:189-224

(1993); Plückthun and Skerra, *Meth. Enzymol.*, 178:497-515 (1989) and in Day, E.D., Advanced Immunochemistry, Second Ed., Wiley-Liss, Inc., New York, NY (1990).

The term **"antigen-presenting cell"** or **"APC"** refers to a heterogeneous group of immune cells that mediate the cellular immune response by processing and presenting antigens for recognition by certain lymphocytes, such as T cells. APCs include, but are not limited to, dendritic cells, macrophages, Langerhans cells and B cells.

The terms **"binds"** or **"binding"** as used herein refer to an interaction between molecules to form a complex which, under physiologic conditions, is relatively stable. Interactions can be, for example, non-covalent interactions including hydrogen bonds, ionic bonds, hydrophobic interactions, and/or van der Waals interactions. A complex can also include the binding of two or more molecules held together by covalent or non-covalent bonds, interactions or forces. The strength of the total non-covalent interactions between a single antigen-binding site on an antibody and a single epitope of a target molecule, such as VISTA, is the affinity of the antibody or functional fragment for that epitope. The ratio of association (k_1) to dissociation (k_{-1}) of an antibody to a monovalent antigen (k_1/ k_{-1}) is the association constant K, which is a measure of affinity. The value of K varies for different complexes of antibody and antigen and depends on both k_1 and k_{-1}. The association constant K for an antibody provided herein can be determined using any method provided herein or any other method well known to those skilled in the art. The affinity at one binding site does not always reflect the true strength of the interaction between an antibody and an antigen. When complex antigens containing multiple, repeating antigenic determinants, such as a polyvalent VISTA, come in contact with antibodies containing multiple binding sites, the interaction of antibody with antigen at one site will increase the probability of a reaction at a second site. The strength of such multiple interactions between a multivalent antibody and antigen is called the avidity. The avidity of an antibody can be a better measure of its binding capacity than is the affinity of its individual binding sites. For example, high avidity can compensate for low affinity as is sometimes found for pentameric IgM antibodies, which can have a lower affinity than IgG, but the high avidity of IgM, resulting from its multivalence, enables it to bind antigen effectively. Methods for determining whether two molecules bind are well known in the art and include, for example, equilibrium dialysis, surface plasmon resonance, and the like. In a particular embodiment, said antibody, or antigen-binding fragment thereof, binds to VISTA with an affinity that is at least two-

fold greater than its affinity for binding to a non-specific molecule such as BSA or casein. In a more particular embodiment, said antibody, or antigen-binding fragment thereof, binds only to VISTA.

As used herein, the term "**biological sample**" or "**sample**" refers to a sample that has been obtained from a biological source, such as a patient or subject. A "biological sample" as used herein refers notably to a whole organism or a subset of its tissues, cells or component parts (*e.g.*, blood vessel, including artery, vein and capillary, body fluids, including but not limited to blood, serum, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). "Biological sample" further refers to a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof. Lastly, "biological sample" refers to a medium, such as a nutrient broth or gel in which an organism has been propagated, which contains cellular components, such as proteins or nucleic acid molecules.

"**Buffer**" refers to a buffer that is resistant to changes in pH due to its conjugate acid-base component. Examples of the buffer which controls the pH in appropriate range include acetate buffer, succinate buffer, gluconate buffer, histidine buffer, oxalate buffer, lactate buffer, phosphate buffer, citrate buffer, tartrate buffer, fumarate buffer, glycylglycine and other organic acid buffers.

Histidine buffer refers to a buffer comprising histidine ions. Examples of histidine buffers include histidine-hydrochloride buffer, histidine-acetate buffer, histidine-phosphate buffer, histidine-sulfate buffer, etc., preferably histidine-hydrochloride buffer. Histidine-hydrochloride buffer is prepared by histidine and hydrochloric acid or by histidine and histidine hydrochloride.

The terms "**cell proliferative disorder**" and "**proliferative disorder**" refer to disorders that are associated with some degree of abnormal cell proliferation. In some embodiments, the cell proliferative disorder is a tumour or cancer. "**Tumour**," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "**cancer**," "**cancerous**," "**cell proliferative disorder**," "**proliferative disorder**" and "tumour" are not mutually exclusive as referred to herein. The terms "**cancer**" and "**cancerous**" refer to or describe the physiological condition in mammals that is typically characterised by unregulated cell growth. A "**cancer**" as used herein is any malignant

neoplasm resulting from the undesired growth, the invasion, and under certain conditions metastasis of impaired cells in an organism. The cells giving rise to cancer are genetically impaired and have usually lost their ability to control cell division, cell migration behaviour, differentiation status and/or cell death machinery. Most cancers form a tumour but some hematopoietic cancers, such as leukaemia, do not. Thus, a "cancer" as used herein may include both benign and malignant cancers. The term "cancer" as used herein refers in particular to any cancer that can be treated by the human antibody of the present disclosure without any limitation. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukaemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (*e.g.* epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, oral cancer, liver cancer, bladder cancer, 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, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, multiple myeloma and B-cell lymphoma, brain cancer, as well as head and neck cancer, and associated metastases. In some embodiments, the cancer is a haematological cancer, which refers to cancer that begins in blood-forming tissue, such as the bone marrow, or in the cells of the immune system. Examples of a haematologic cancer are leukaemia (*e.g.*, acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myelogenous leukaemia (CML), chronic lymphocytic leukaemia (CLL), or acute monocytic leukaemia (AMoL)), lymphoma (Hodgkin lymphoma or non-Hodgkin lymphoma), and myeloma (multiple myeloma, plasmacytoma, localised myeloma or extramedullary myeloma).

A "**chemotherapeutic agent**" is a chemical or biological agent (*e.g.*, an agent, including a small molecule drug or biologic, such as an antibody or cell) useful in the treatment of cancer, regardless of mechanism of action. Chemotherapeutic agents include compounds used in targeted therapy and conventional chemotherapy. Chemotherapeutic agents include, but are not limited to, alkylating agents, anti-

metabolites, anti-tumour antibiotics, mitotic inhibitors, chromatin function inhibitors, anti-angiogenesis agents, anti-oestrogens, anti-androgens or immunomodulators.

As used herein, a "**CDR**" refers to one of three hypervariable regions (H1, H2 or H3) within the non-framework region of the immunoglobulin (Ig or antibody) VH β-sheet framework, or one of three hypervariable regions (L1, L2 or L3) within the non-framework region of the antibody VL β-sheet framework. Accordingly, CDRs are variable region sequences interspersed within the framework region sequences. CDR regions are well known to those skilled in the art and have been defined by, for example, Kabat as the regions of most hypervariability within the antibody variable (V) domains (Kabat *et al.* (1977) *J. Biol. Chem.* **252**:6609-6616; Kabat (1978) *Adv. Prot. Chem.* **32**:1-75). The Kabat CDRs are based on sequence variability and are the most commonly used (Kabat *eta/.* (1991) *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD). Chothia refers instead to the location of the structural loops (Chothia and Lesk (1987) *J Mol. Biol.* **196**:901-917). CDR region sequences also have been defined structurally by Chothia as those residues that are not part of the conserved β-sheet framework, and thus are able to adopt different conformations (Chothia and Lesk (1987) *J. Mol. Biol.* **196**:901-917). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). Both terminologies are well recognised in the art. CDR region sequences have also been defined by AbM, Contact and IMGT. The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modelling software. The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. Recently, a universal numbering system has been developed and widely adopted, ImMunoGeneTics (IMGT) Information System® (Lefranc *et al.* (2003) *Dev. Comp. Immunol.* **27**(1):55-77). The IMGT universal numbering has been defined to compare the variable domains whatever the antigen receptor, the chain type, or the species [Lefranc M.-P. (1997) *Immunol. Today* **18**: 509; Lefranc M.-P. (1999) *The Immunologist* **7**: 132-136]. In the IMGT universal numbering, the conserved amino acids always have the same position, for instance cysteine 23 (1st-CYS), tryptophan 41 (CONSERVED-TRP),

hydrophobic amino acid 89, cysteine 104 (2nd-CYS), phenylalanine or tryptophan 118 (J-PHE or J-TRP). The IMGT universal numbering provides a standardised delimitation of the framework regions (FR1-IMGT: positions 1 to 26, FR2-IMGT: 39 to 55, FR3-IMGT: 66 to 104 and FR4-IMGT: 118 to 128) and of the complementarity determining regions: CDR1-IMGT: 27 to 38, CDR2-IMGT: 56 to 65 and CDR3-IMGT: 105 to 117. As gaps represent unoccupied positions, the CDR-IMGT lengths (shown between brackets and separated by dots, *e.g.* [8.8.13]) become crucial information. The IMGT universal numbering is used in 2D graphical representations, designated as IMGT Colliers de Perles [Ruiz, M. and Lefranc, M.-P., *Immunogenetics*, **53**: 857-883 (2002); Kaas, Q. and Lefranc, M.-P., *Current Bioinformatics*, **2**: 21-30 (2007)], and in 3D structures in IMGT/3Dstructure-DB [Kaas, Q., Ruiz, M. and Lefranc, M.-P., T cell receptor and MHC structural data. *Nucl. Acids. Res.*, **32**: D208-D210 (2004)]. The positions of CDRs within a canonical antibody variable domain have been determined by comparison of numerous structures (Al-Lazikani *et al.*, *J. Mol. Biol.* 273:927-948 (1997); Morea *et al.*, *Methods* 20:267-279 (2000)). Because the number of residues within a hypervariable region varies in different antibodies, additional residues relative to the canonical positions are conventionally numbered with a, b, c and so forth next to the residue number in the canonical variable domain numbering scheme (Al-Lazikani *et al.*, *supra* (1997)). Such nomenclature is similarly well known to those skilled in the art.

Hypervariable regions may comprise "**extended hypervariable regions**" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 or 26-35A (H1), 50-65 or 49-65 (H2) and 93-102, 94-1 02, or 95-102 (H3) in the VH. The variable domain residues are 25 numbered according to Kabat *et al.*, *supra*, for each of these definitions. As used herein, the terms "**HVR**" and "**CDR**" are used interchangeably.

As used herein, a "**checkpoint inhibitor**" refers to a molecule, such as *e.g.*, a small molecule, a soluble receptor, or an antibody, which targets an immune checkpoint and blocks the function of said immune checkpoint. More specifically, a "checkpoint inhibitor" as used herein is a molecule, such as *e.g.*, a small molecule, a soluble receptor, or an antibody, that is capable of inhibiting or otherwise decreasing one or more of the biological activities of an immune checkpoint. In some embodiments, an inhibitor of an immune checkpoint protein (*e.g.*, an antagonistic anti-VISTA antibody provided herein) can, for example, act by inhibiting or otherwise decreasing the activation and/or cell signalling pathways of the cell expressing said

immune checkpoint protein (*e.g.*, a T cell), thereby inhibiting a biological activity of the cell relative to the biological activity in the absence of the antagonist. Example of immune checkpoint inhibitors include small molecule drugs, soluble receptors, and antibodies.

The term "**complement-dependent cytotoxicity**" or "**CDC**" as used herein refers to the process of antibody-mediated complement activation resulting in the lysis of a cell according to the mechanism outlined above upon binding of the antibody to its antigen located on that cell. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.*, an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.*, as described in Gazzano-Santaro et al., J. Immunol. Methods, 202:163 (1996), may be performed. In the art normal human serum is used as a complement source.

The term "**constant region**" or "**constant domain**" refers to a carboxy terminal portion of the light and heavy chain which is not directly involved in binding of the antibody to antigen but exhibits various effector function, such as interaction with the Fc receptor. The terms refer to the portion of an immunoglobulin molecule having a more conserved amino acid sequence relative to the other portion of the immunoglobulin, the variable domain, which contains the antigen binding site. The constant domain contains the CH1, CH2 and CH3 domains of the heavy chain and the CL domain of the light chain.

As described herein, a "**cytotoxic agent**" refers to an agent which, when administered to a subject, treats or prevents the development of cell proliferation, preferably the development of cancer in the subject's body, by inhibiting or preventing a cellular function and/or causing cell death. The cytotoxic agent that can be used in the present antibody-drug conjugate includes any agent, part thereof, or residue having cytotoxic effect or inhibitory effect on cell proliferation. Examples of such agents include (i) chemotherapeutic agent capable of functioning as a microtubulin inhibitor, a mitotic inhibitor, a topoisomerase inhibitor, or a DNA interchelator; (ii) protein toxin capable of functioning enzymatically; and (iii) radioisotopes (radioactive nuclide). The cytotoxic agent may be conjugated to an antibody, such as *e.g.*, an anti-VISTA antibody, to form an immunoconjugate. Preferably, the cytotoxic agent is released from the antibody under specific conditions, *e.g.*, under acidic conditions,

thereby affecting therapeutically the target cells, *e.g.*, by preventing the proliferation thereof or by displaying a cytotoxic effect.

The term "**decreased**", as used herein, refers to the activity of a protein, *e.g.*, VISTA, at least 1-fold (*e.g.*, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 1000, 10,000- fold or more) lower than its reference value. "Decreased", as it refers to the activity of a protein, *e.g.*, VISTA, of a subject, signifies also at least 5% lower (*e.g.*, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%), 95%), 99%), or 100%) than the activity of the protein in the reference sample or with respect to the reference value for said protein. The term "decreased", as used herein, also refers to the level of a biomarker, *e.g.*, VISTA, of a subject at least 1-fold (*e.g.*, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 1000, 10,000- fold or more) lower than its reference value. "Decreased", as it refers to the level of a biomarker, *e.g.*, VISTA, of a subject, signifies also at least 5% lower (*e.g.*, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%), 95%), 99%), or 100%) than the level in the reference sample or with respect to the reference value for said marker.

The term "**detecting**" as used herein encompasses quantitative or qualitative detection.

The term "**detectable probe**," as used herein, refers to a composition that provides a detectable signal. The term includes, without limitation, any fluorophore, chromophore, radiolabel, enzyme, antibody or antibody fragment, and the like, that provide a detectable signal via its activity.

In the context of a polypeptide, the term "**derivative**" as used herein refers to a polypeptide that comprises an amino acid sequence of a VISTA polypeptide, a fragment of a VISTA polypeptide, or an antibody that binds to a VISTA polypeptide which has been altered by the introduction of amino acid residue substitutions, deletions or additions. The term "derivative" as used herein also refers to a VISTA polypeptide, a fragment of a VISTA polypeptide, or an antibody that binds to a VISTA polypeptide which has been chemically modified, *e.g.*, by the covalent attachment of any type of molecule to the polypeptide. For example, but not by way of limitation, a VISTA polypeptide, a fragment of a VISTA polypeptide, or a VISTA antibody may be chemically modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation,

derivatisation by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* The derivatives are modified in a manner that is different from naturally occurring or starting peptide or polypeptides, either in the type or location of the molecules attached. Derivatives further include deletion of one or more chemical groups which are naturally present on the peptide or polypeptide. A derivative of a VISTA polypeptide, a fragment of a VISTA polypeptide, or a VISTA antibody may be chemically modified by chemical modifications using techniques known to those of skill in the art, including, but not limited to specific chemical cleavage, acetylation, formulation, metabolic synthesis of tunicamycin, *etc.* Further, a derivative of a VISTA polypeptide, a fragment of a VISTA polypeptide, or a VISTA antibody may contain one or more non-classical amino acids. A polypeptide derivative possesses a similar or identical function as a VISTA polypeptide, a fragment of a VISTA polypeptide, or a VISTA antibody described herein.

The term "diagnostic agent" refers to a substance administered to a subject that aids in the diagnosis of a disease. Such substances can be used to reveal, pinpoint, and/or define the localisation of a disease-causing process. In some embodiments, a diagnostic agent includes a substance that is conjugated to an antibody provided herein, that when administered to a subject or contacted to a sample from a subject, aids in the diagnosis of cancer, tumour formation, or any other VISTA-mediated disease, disorder or condition.

The term "**detectable agent**" refers to a substance that can be used to ascertain the existence or presence of a desired molecule, such as an antibody provided herein, in a sample or subject. A detectable agent can be a substance that is capable of being visualised or a substance that is otherwise able to be determined and/or measured (*e.g.*, by quantitation).

The term "**detecting**" as used herein encompasses quantitative or qualitative detection.

An "**effective amount**" or "**therapeutically effective amount**" of an agent, *e.g.*, a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to elicit the desired biological response in a subject. Such response includes alleviation of the symptoms of the disease or disorder being treated, prevention, inhibition or a delay in the recurrence of symptom of the disease or of the

disease itself, an increase in the longevity of the subject compared with the absence of the treatment, or prevention, inhibition or delay in the progression of symptom of the disease or of the disease itself. An "effective amount" is in particular the amount of the agent effective to achieve the desired therapeutic or prophylactic result. More specifically, an "effective amount" as used herein is an amount of the agent that confers a therapeutic benefit. A therapeutically effective amount is also one in which any toxic or detrimental effects of the agent are outweighed by the therapeutically beneficial effects.

An effective amount can be administered in one or more administrations, applications or dosages. Such delivery is dependent on a number of variables including the time period for which the individual dosage unit is to be used, the bioavailability of the agent, the route of administration, *etc.* In some embodiments, effective amount also refers to the amount of an antibody (*e.g.*, an anti-VISTA antibody) provided herein to achieve a specified result (*e.g.*, inhibition of an immune checkpoint biological activity, such as modulating T cell activation). In some embodiments, this term refers to the amount of a therapy (*e.g.*, an immune checkpoint inhibitor such as *e.g.*, an anti-VISTA antibody) which is sufficient to reduce and/or ameliorate the severity and/or duration of a given disease, disorder or condition and/or a symptom related thereto. This term also encompasses an amount necessary for the reduction or amelioration of the advancement or progression of a given disease, disorder or condition, reduction or amelioration of the recurrence, development or onset of a given disease, disorder or condition, and/or to improve or enhance the prophylactic or therapeutic effect(s) of another therapy (*e.g.*, a therapy other than said immune checkpoint inhibitor). In the context of cancer therapy, a therapeutic benefit means for example any amelioration of cancer, including any one of, or combination of, halting or slowing the progression of cancer (*e.g.*, from one stage of cancer to the next), halting or delaying aggravation or deterioration of the symptoms or signs of cancer, reducing the severity of cancer, inducing remission of cancer, inhibiting tumour cell proliferation, tumour size, or tumour number, or reducing levels of biomarker(s) indicative of the cancer. In some embodiments, the effective amount of an antibody is from about 0.1 mg/kg (mg of antibody per kg weight of the subject) to about 100 mg/kg. In some embodiments, an effective amount of an antibody provided therein is about 0.1 mg/kg, about 0.5 mg/kg, about 1 mg/kg, 3 mg/kg, 5 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, about 30 mg/kg, about 35 mg/kg, about 40 mg/kg, about 45 mg/kg,

about 50 mg/kg, about 60 mg/kg, about 70 mg/kg, about 80 mg/kg about 90 mg/kg or about 100 mg/kg (or a range therein).

As used herein, the term "**effector functions**" refer to biological functions carried by the Fc domain of an immunoglobulin (e.g., the anti-VISTA antibody described herein). These Fc domain-mediated activities are mediated via immunological effector cells, such as killer cells, natural killer cells, and activated macrophages, or various complement components. These effector functions involve activation of receptors on the surface of said effector cells, through the binding of the Fc domain of an antibody to the said receptor or to complement component(s). "Effector functions" as used herein encompass such activities as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC).

"**Effector cells**" as used herein refer to leukocytes which express one or more FcRs and perform effector functions. The cells express at least FcγRI, FCγRII, FcγRIII and/or FcγRIV and carry out ADCC effector function. FcR expression on hematopoietic cells is summarised in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils.

The term "**encode**" or grammatical equivalents thereof as it is used in reference to nucleic acid molecule refers to a nucleic acid molecule in its native state or when manipulated by methods well known to those skilled in the art that can be transcribed to produce mRNA, which is then translated into a polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid molecule, and the encoding sequence can be deduced therefrom.

The term "**epitope**" as used herein refers to the region of an antigen, such as VISTA polypeptide or VISTA polypeptide fragment, to which an antibody binds. Preferably, an epitope as used herein is a localised region on the surface of an antigen, such as VISTA polypeptide or VISTA polypeptide fragment, that is capable of being bound to one or more antigen binding regions of an antibody, and that has antigenic or immunogenic activity in an animal, such as a mammal (*e.g.*, a human), that is capable of eliciting an immune response. An epitope having immunogenic activity is a

portion of a polypeptide that elicits an antibody response in an animal. An epitope having antigenic activity is a portion of a polypeptide to which an antibody binds as determined by any method well known in the art, for example, by an immunoassay. Antigenic epitopes need not necessarily be immunogenic. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three-dimensional structural characteristics as well as specific charge characteristics. An epitope can be formed by contiguous residues or by non-contiguous residues brought into close proximity by the folding of an antigenic protein. Epitopes formed by contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by non-contiguous amino acids are typically lost under said exposure. In some embodiments, a VISTA epitope is a three-dimensional surface feature of a VISTA polypeptide. In other embodiments, a VISTA epitope is linear feature of a VISTA polypeptide. Generally, an antigen has several or many different epitopes and reacts with many different antibodies.

The term "**excipient**" as used herein refers to an inert substance which is commonly used as a diluent, vehicle, preservative, binder, or stabilising agent for drugs which imparts a beneficial physical property to a formulation, such as increased protein stability, increased protein solubility, and decreased viscosity. Examples of excipients include, but are not limited to, proteins (*e.g.*, serum albumin, *etc.*), amino acids (*e.g.*, aspartic acid, glutamic acid, lysine, arginine, glycine, histidine, *etc.*), fatty acids and phospholipids (*e.g.*, alkyl sulfonates, caprylate, *etc.*), surfactants (*e.g.*, SDS, polysorbate, non-ionic surfactant, *etc.*), saccharides (*e.g.*, sucrose, maltose, trehalose, *etc.*) and polyols (*e.g.*, mannitol, sorbitol, *etc.*). See, also, Remington's Pharmaceutical Sciences (1990) Mack Publishing Co., Easton, PA, which is hereby incorporated by reference in its entirety.

The term "**framework**" or "**FR**" residues refers to those variable domain residues other than the hypervariable region residues herein defined. FR residues are those variable domain residues flanking the CDRs. FR residues are present, *e.g.*, in chimeric, Humanised, human, domain antibodies, diabodies, linear antibodies, and bispecific antibodies.

The term "**heavy chain**" when used in reference to an antibody refers to a polypeptide chain of about 50-70 kDa, wherein the amino-terminal portion includes a variable region of about 120 to 130 or more amino acids and a carboxy-terminal portion

that includes a constant region. The constant region can be one of five distinct types, referred to as alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ), based on the amino acid sequence of the heavy chain constant region. The distinct heavy chains differ in size: α, δ and γ contain approximately 450 amino acids, while μ and ε contain approximately 550 amino acids. When combined with a light chain, these distinct types of heavy chains give rise to five well known classes of antibodies, IgA, IgD, IgE, IgG and IgM, respectively, including four subclasses of IgG, namely IgG1, IgG2, IgG3 and IgG4. A heavy chain can be a human heavy chain.

The term "**hinge region**" refers herein to a flexible amino acid stretch in the central part of the heavy chains of the IgG and IgA immunoglobulin classes, which links these 2 chains by disulfide bonds. The hinge region is generally defined as stretching from Glu216 to Pro230 of human IgG1 (Burton, Mol Immunol, 22: 161-206, 1985). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S bonds in the same positions. The "CH2 domain" of a human IgG Fc portion (also referred to as "Cγ2" domain) usually extends from about amino acid 231 to about amino acid 340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilise the CH2 domain (Burton, Mol Immunol, 22: 161-206, 1985). The "CH3 domain" comprises the stretch of residues C- terminal to a CH2 domain in an Fc portion (*i.e.*, from about amino acid residue 341 to about amino acid residue 447 of an IgG).

The term "**host**" as used herein refers to an animal, such as a mammal (*e.g.*, a human).

The term "**host cell**" as used herein refers to the particular subject cell transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome.

A "**humanised antibody**" refers to a chimeric antibody that contains minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanised antibody is a human immunoglobulin (recipient antibody) in which residues from a CDR of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, some of the skeleton segment residues (called FR for framework) can be modified to preserve binding affinity, according to techniques known by a man skilled in the art (Jones *et al.*, Nature, 321:522-525, 1986). In some embodiments, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. In certain embodiments, a humanised antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanised antibody optionally may comprise at least a portion of an antibody constant region (Fc), typically that of a human immunoglobulin. A "humanised form" of an antibody, *e.g.*, a non-human antibody, refers to an antibody that has undergone humanisation. The goal of humanisation is a reduction in the immunogenicity of a xenogenic antibody, such as a murine antibody, for introduction into a human, while maintaining the full antigen binding affinity and specificity of the antibody. For further details, see, *e.g.*, Jones et al, Nature 321: 522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992). See also, *e.g.*, Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1 :105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

As used herein, "**identifying**" as it refers to a subject that has a condition refers to the process of assessing a subject and determining that the subject has a condition, for example, suffers from cancer.

As used herein, the terms "**immune checkpoint**" or "**immune checkpoint protein**" refer to certain proteins made by some types of immune system cells, such as T cells, and some cancer cells. Such proteins regulate T cell function in the immune system. Notably, they help keep immune responses in check and can keep T cells from killing cancer cells. Said immune checkpoint proteins achieve this result by interacting with specific ligands which send a signal into the T cell and essentially switch off or

inhibit T cell function. Inhibition of these proteins results in restoration of T cell function and an immune response to the cancer cells. Examples of checkpoint proteins include, but are not limited to CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIGIT, TIM3, GAL9, LAG3, VSIG4, KIR, 2B4 (belongs to the CD2 family of molecules and is expressed on all NK, γδ, and memory CD8+ (αß) T cells), CD160 (also referred to as BY55), CGEN-15049, CHK1 and CHK2 kinases, IDO1, A2aR, and various B7 family ligands.

The term "**increased**", as used herein, refers to the activity of a protein, *e.g.*, VISTA, at least 1-fold (*e.g.* 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 1000, 10,000-fold or more) greater than its reference value. "Increased", as it refers to the activity of a protein, *e.g.*, VISTA, of a subject, signifies also at least 5% greater (*e.g.*, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100%) than the activity of the protein in the reference sample or with respect to the reference value for said protein. The term "increased", as used herein, also refers to the level of a biomarker, *e.g.*, VISTA, of a subject at least 1-fold (*e.g.*, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 1000, 10,000-fold or more) greater than its reference value. "Increased", as it refers to the level of a biomarker, *e.g.*, VISTA, of a subject, signifies also at least 5% greater (*e.g.*, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100%) than the level in the reference sample or with respect to the reference value for said marker.

The term "**inhibit**," or "**block**", or a grammatical equivalent thereof, when used in the context of an antibody refers to an antibody that suppresses, restrains or decreases a biological activity of the antigen to which the antibody binds. The inhibitory effect of an antibody can be one which results in a measurable change in the antigen's biological activity. In particular instances, "inhibit," or "block" refers to an antibody that prevents or stops a biological activity of the antigen to which the antibody binds. A blocking antibody includes an antibody that combines with an antigen without eliciting a reaction, but that blocks another protein from later combining or complexing with that antigen. The blocking effect of an antibody can be one which results in a measurable change in the antigen's biological activity. In some embodiments, an anti-VISTA antibody described herein blocks the ability of VISTA to bind VSIG3, which can result in inhibiting or blocking suppressive signals of VISTA. Certain anti-VISTA antibodies described herein inhibit or block suppressive signals of VISTA on VISTA-expressing cells, including by about 98% to about 100% as compared to

the appropriate control (*e.g.*, the control being cells not treated with the antibody being tested). In some embodiments, the anti-VISTA antibody described herein blocks the binding of the extracellular domain VISTA to VSIG3 and/or blocks the binding of a VISTA-expressing cell to a VSIG3-expressing cell. In some embodiments, an anti-VISTA antibody described herein blocks the ability of VISTA to bind PSGL-1, preferably at acidic pH (pH between 5.9 and 6.5), which can result in inhibiting or blocking suppressive signals of VISTA. Certain anti-VISTA antibodies described herein inhibit or block suppressive signals of VISTA on VISTA-expressing cells, including by about 98% to about 100% as compared to the appropriate control (*e.g.*, the control being cells not treated with the antibody being tested). In some embodiments, the anti-VISTA antibody described herein blocks, preferably at acidic pH (pH between 5.9 and 6.5), the binding of the extracellular domain VISTA to PSGL-1 and/or blocks, preferably at acidic pH (pH between 5.9 and 6.5), the binding of a VISTA-expressing cell to a PSGL-1-expressing cell.

The term "**immune infiltrate**" or "**tumour immune cells**" refers to cells that infiltrate the microenvironment of a tumour, including, but not limited to, lymphocytes (*e.g.*, T cells, B-cells, natural killer (NK) cells), dendritic cells, mast cells, and macrophages.

As used herein, the term "**in combination**" in the context of the administration of other therapies refers to the use of more than one therapy (*e.g.*, an anti-VISTA antibody and an immune checkpoint inhibitor such as an anti-PD-1 antibody or an anti-PD-L1 antibody). The use of the term "**in combination**" does not restrict the order or the time in which therapies are administered to a subject (*e.g.*, one therapy before, concurrent with, or after another therapy). A first therapy can be administered before (*e.g.*, 1 minute, 45 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks), concurrently, or after (*e.g.*, 1 minute, 45 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks) the administration of a second therapy to a subject which had, has, or is susceptible to a VISTA-mediated disease, disorder or condition. Any additional therapy can be administered in any order or time with the other additional therapies (*e.g.*, an anti-VISTA antibody and an immune checkpoint inhibitor such as an anti-PD-1 antibody or an anti-PD-L1 antibody). In some embodiments, the antibodies

can be administered in combination with one or more therapies (*e.g.*, therapies that are not the antibodies that are currently administered to prevent, treat, manage, and/or ameliorate a VISTA-mediated disease, disorder or condition). Non-limiting examples of therapies that can be administered in combination with an antibody include an antagonist to a co-inhibitory molecule, an agonist to a co-stimulatory molecule, a chemotherapeutic agent, radiation, analgesic agents, anaesthetic agents, antibiotics, or immunomodulatory agents or any other agent listed in the U.S. Pharmacopoeia and/or Physician's Desk Reference.

An "**isolated**" antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoresis (*e.g.*, SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatography (*e.g.*, ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, *e.g.*, Flatman et al., J. Chromatogr. B 848:79-87 (2007).

An "**isolated**" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

The term "K_D" used herein means a dissociation constant of a specific antibody-antigen interaction and is used as an indicator for measuring the affinity of an antibody for an antigen. Lower K_D means higher affinity of an antibody for an antigen.

As intended herein, the "**level**" of a biomarker, *e.g.*, VISTA, consists of a quantitative value of the biomarker in a sample, *e.g.*, in a sample collected from a cancer-suffering patient. In some embodiments, the quantitative value does not consist of an absolute value that is actually measured, but rather consists of a final value resulting from taking into consideration of a signal to noise ratio occurring with the assay format used, and/or taking into consideration of calibration reference values that are used to increase reproducibility of the measures of the level of a cancer marker, from assay-to-assay. In some embodiments, the "level" of a biomarker, *e.g.*, VISTA, is expressed as arbitrary units, since what is important is that the same kind of arbitrary units are compared (i) from assay-to-assay, or (ii) from one cancer-suffering patient to others, or (iii) from assays performed at distinct time periods for the same

patient, or (iv) between the biomarker level measured in a patient's sample and a predetermined reference value (which may also be termed a "cut-off" value herein).

The term "**light chain**" when used in reference to an antibody refers to a polypeptide chain of about 25 kDa, wherein the amino-terminal portion includes a variable region of about 100 to about 110 or more amino acids and a carboxy-terminal portion that includes a constant region. The approximate length of a light chain is 211 to 217 amino acids. There are two distinct types, referred to as kappa (κ) of lambda (λ) based on the amino acid sequence of the constant domains. Light chain amino acid sequences are well known in the art. A light chain can be a human light chain.

As used herein, the term "**monoclonal antibody**" designates an antibody arising from a nearly homogeneous antibody population, wherein population comprises identical antibodies except for a few possible naturally-occurring mutations which can be found in minimal proportions. A monoclonal antibody arises from the growth of a single cell clone, such as a hybridoma, and is characterised by heavy chains of one class and subclass, and light chains of one type. As used herein, a monoclonal antibody shows specific binding to a single antigenic site (*i.e.*, single epitope) when the antibody is presented to it. The monoclonal antibody can be produced by various methods that are well known in the corresponding technical area.

The term "**native**" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to those which are found in nature and not manipulated by a human being.

As used herein, the "**percentage identity**" or "**% identity**" between two sequences of nucleic acids or amino acids refers to the percentage of identical nucleotides or amino acid residues between the two sequences to be compared, obtained after optimal alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly along their length. The comparison of two nucleic acid or amino acid sequences is traditionally carried out by comparing the sequences after having optimally aligned them, said comparison being able to be conducted by segment or by using an "alignment window". Optimal alignment of the sequences for comparison can be carried out, in addition to comparison by hand, by means of methods known by a man skilled in the art.

For the amino acid sequence exhibiting at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with a reference amino acid sequence, preferred examples include those containing the reference sequence, certain modifications, notably a deletion, addition or substitution of at least one amino acid, truncation or extension. In the case of substitution of one or more consecutive or non-consecutive amino acids, substitutions are preferred in which the substituted amino acids are replaced by "equivalent" amino acids. Here, the expression "equivalent amino acids" is meant to indicate any amino acids likely to be substituted for one of the structural amino acids without however modifying the biological activities of the corresponding antibodies and of those specific examples defined below. Equivalent amino acids can be determined either on their structural homology with the amino acids for which they are substituted or on the results of comparative tests of biological activity between the various antibodies likely to be generated.

As a non-limiting example, Table 1 below summarises the possible substitutions likely to be carried out without resulting in a significant modification of the biological activity of the corresponding modified antigen binding protein; inverse substitutions are naturally possible under the same conditions.

Table 1

Original residue	Substitution(s)
Ala (A)	Val, Gly, Pro
Arg (R)	Lys, His
Asn (N)	Gln
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn

Glu (E)	Asp
Gly (G)	Ala
His (H)	Arg
Ile (I)	Leu
Leu (L)	Ile, Val, Met
Lys (K)	Arg
Met (M)	Leu
Phe (F)	Tyr
Pro (P)	Ala
Ser (S)	Thr, Cys
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Phe, Trp
Val (V)	Leu, Ala

The term "**pharmaceutically acceptable**" as used herein means being approved by a regulatory agency of the Federal or a state government, or listed in the U.S. Pharmacopeia, European Pharmacopeia or other generally recognised Pharmacopeia for use in animals, and more particularly in humans. More specifically, when referring to a carrier, the expression "**pharmaceutically acceptable**" means that the carrier(s) is compatible with the other ingredient(s) of the composition and is not deleterious to the recipient thereof. Accordingly, as used herein, the expression "**pharmaceutically acceptable carrier**" refers to a carrier or a diluent which does not inhibit the biological activity and characteristics of a compound for administration without stimulating a living organism. The type of carrier can be selected based upon the intended route of

administration. The amount of each carriers used may vary within ranges conventional in the art. As a pharmaceutically acceptable carrier in the composition which is prepared as a liquid solution, physiological saline, sterilised water, buffered saline, albumin injection solution, dextrose solution, maltodextrin solution, glycerol, and a mixture of one or more of them can be used as a sterilised carrier suitable for a living organism. If necessary, common additives like anti-oxidant, buffer solution, and bacteriostat may be added. Furthermore, by additionally adding a diluent, a dispersant, a surfactant, a binder, or a lubricant, the composition can be prepared as a formulation for injection like aqueous solution, suspension, and emulsion, a pill, a capsule, a granule, or a tablet.

As used herein, the term "**polyclonal antibody**" refers to an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In general, polyclonal antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes producing non-identical antibodies. Usually, polyclonal antibodies are obtained directly from an immunised animal.

As used herein, the term "**polynucleotide**," "**nucleotide**," **nucleic acid**" "**nucleic acid molecule**" and other similar terms are used interchangeable and include DNA, RNA, mRNA and the like.

The term "**radiation**," when used in the therapeutic context refers to a type of treatment that uses a beam of intense energy to kill target cells (*e.g.*, cancer cells). Radiation therapy includes the use of X-rays, protons or other forms of energy that are administered through an external beam. Radiation therapy also includes radiation treatment that is placed into a patient's body (*e.g.*, brachytherapy) whereby a small container of radioactive material is implanted directly into or near a tumour.

The term "**reference value**", as used herein, refers to the expression level of a biomarker under consideration (*e.g.*, VISTA) in a reference sample. A "**reference sample**", as used herein, means a sample obtained from subjects, preferably two or more subjects, known to be free of the disease or, alternatively, from the general population. The suitable reference expression levels of biomarker can be determined by measuring the expression levels of said biomarker in several suitable subjects, and such reference levels can be adjusted to specific subject populations. The reference value or reference level can be an absolute value; a relative value; a value that has an

upper or a lower limit; a range of values; an average value; a median value, a mean value, or a value as compared to a particular control or baseline value. A reference value can be based on an individual sample value such as, for example, a value obtained from a sample from the subject being tested, but at an earlier point in time. The reference value can be based on a large number of samples, such as from population of subjects of the chronological age matched group, or based on a pool of samples including or excluding the sample to be tested.

As used herein, the term "**side effects**" encompasses unwanted and adverse effects of a therapy (*e.g.*, a prophylactic or therapeutic agent). Unwanted effects are not necessarily adverse. An adverse effect from a therapy (*e.g.*, a prophylactic or therapeutic agent) might be harmful or uncomfortable or risky. Examples of side effects include, diarrhoea, cough, gastroenteritis, wheezing, nausea, vomiting, anorexia, abdominal cramping, fever, pain, loss of body weight, dehydration, alopecia, dyspnoea, insomnia, dizziness, mucositis, nerve and muscle effects, fatigue, dry mouth, and loss of appetite, rashes or swellings at the site of administration, flu-like symptoms such as fever, chills and fatigue, digestive tract problems and allergic reactions. Additional undesired effects experienced by patients are numerous and known in the art. Many are described in the Physician's Desk Reference (67^{th} ed., 2013).

The terms "**stability**" and "**stable**" as used herein in the context of a liquid formulation comprising an antibody (including antibody fragment thereof) that specifically binds to an antigen of interest (*e.g.*, VISTA) refer to the resistance of the antibody (including antibody fragment thereof) in the formulation to aggregation, degradation or fragmentation under given manufacture, preparation, transportation and storage conditions. The "stable" formulations of the disclosure retain biological activity under given manufacture, preparation, transportation and storage conditions. The stability of said antibody (including antibody fragment thereof) can be assessed by degrees of aggregation, degradation or fragmentation, as measured by HPSEC, reverse phase chromatography, static light scattering (SLS), Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), urea unfolding techniques, intrinsic tryptophan fluorescence, differential scanning calorimetry, and/or ANS binding techniques, compared to a reference formulation. For example, a reference formulation may be a reference standard frozen at -70°C consisting of 20 mg/ml of an antibody (including antibody fragment thereof) (for

example, but not limited to, an antibody comprising a heavy chain sequence of SEQ ID NO:21, a light chain sequence of SEQ ID NO:22) in 25 mM histidine, pH 6.5 that contains 150 mM NaCl, and 0.006% polysorbate 80 (v/v), which reference formulation regularly gives a single monomer peak (*e.g.*, ≥ 97% area) by HPSEC. The overall stability of a formulation comprising an antibody (including antibody fragment thereof) can be assessed by various immunological assays including, for example, ELISA and radioimmunoassay using isolated antigen molecules.

A "**subject**" which may be subjected to the methodology described herein may be any of mammalian animals including human, dog, cat, cattle, goat, pig, swine, sheep and monkey. A human subject can be known as a patient. In one embodiment, "**subject**" or "**subject in need**" refers to a mammal that is suffering from cancer or is suspected of suffering from cancer or has been diagnosed with cancer. As used herein, a "**cancer-suffering subject**" refers to a mammal that is suffering from cancer or has been diagnosed with cancer. A "**control subject**" refers to a mammal that is not suffering from cancer, and is not suspected of suffering from cancer.

As used herein "**substantially all**" refers to refers to at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99%, or about 100%.

As used herein, the term "**therapeutic agent**" refers to any agent that can be used in treating, preventing or alleviating a disease, disorder or condition, including in the treatment, prevention or alleviation of one or more symptoms of a VISTA-mediated disease, disorder, or condition and/or a symptom related thereto. In some embodiments, a therapeutic agent refers to an anti-VISTA antibody provided herein. In some embodiments, a therapeutic agent refers to an agent other than an anti-VISTA antibody provided herein. In some embodiments, a therapeutic agent is an agent which is known to be useful for, or has been or is currently being used for the treatment, prevention or alleviation of one or more symptoms of a VISTA-mediated disease, disorder, condition, and/or a symptom related thereto.

The combination of therapies (*e.g.*, use of therapeutic agents) can be more effective than the additive effects of any two or more single therapies. For example, a synergistic effect of a combination of therapeutic agents permits the use of lower dosages of one or more of the agents and/or less frequent administration of the agents

to a subject with a VISTA-mediated disease, disorder or condition and/or a symptom related thereto. The ability to utilise lower dosages of therapeutic therapies and/or to administer the therapies less frequently reduces the toxicity associated with the administration of the therapies to a subject without reducing the efficacy of the therapies in the prevention, treatment or alleviation of one or more symptoms of a VISTA-mediated disease, disorder or condition and/or a symptom related thereto. In addition, a synergistic effect can result in improved efficacy of therapies in the prevention, treatment or alleviation of one or more symptoms of a VISTA-mediated disease, disorder or condition and/or a symptom related thereto. Finally, synergistic effect of a combination of therapies (*e.g.*, therapeutic agents) may avoid or reduce adverse or unwanted side effects associated with the use of any single therapy.

The term "**therapeutically effective amount**" as used herein refers to the amount of a therapeutic agent (*e.g.*, an anti-VISTA antibody or any other therapeutic agent, including as described herein, including, for example, an immune checkpoint inhibitor, such as *e.g.*, an anti-PD-1 antibody or an anti-PD-L1 antibody) that is sufficient to reduce and/or ameliorate the severity and/or duration of a given disease, disorder or condition and/or a symptom related thereto. A therapeutically effective amount of a therapeutic agent can be an amount necessary for the reduction or amelioration of the advancement or progression of a given disease, disorder or condition, reduction or amelioration of the recurrence, development or onset of a given disease, disorder or condition and/or to improve or enhance the prophylactic or therapeutic effect of another therapy (*e.g.*, a therapy other than the administration of an anti-VISTA antibody, including as described herein).

As used herein, the term "**therapy**" refers to any protocol, method and/or agent that can be used in the prevention, management, treatment and/or amelioration of a VISTA-mediated disease, disorder or condition. In some embodiments, the terms "**therapies**" and "**therapy**" refer to a biological therapy, supportive therapy, and/or other therapies useful in the treatment, prevention and/or amelioration of a VISTA-mediated disease, disorder or condition known to one of skill in the art such as medical personnel.

As used herein, "**treating**" a disease in a subject or "**treating**" a subject having a disease refers to subjecting the subject to a pharmaceutical treatment, *e.g.*, the administration of a drug, such that the extent of the disease is decreased or prevented.

For example, treating results in the reduction of at least one sign or symptom of the disease or condition. Treatment includes (but is not limited to) administration of a composition, such as a pharmaceutical composition, and may be performed either prophylactically, or subsequent to the initiation of a pathologic event. Treatment can require administration of an agent and/or treatment more than once. In some embodiments, such terms refer to the reduction or amelioration of the progression, severity, and/or duration of a disease, that is responsive to immune modulation, such modulation resulting from increasing T cell activation.

The term "**tumour microenvironment**" refers to the cellular environment in which a tumour exists. A tumour microenvironment can include surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signalling molecules and the extracellular matrix.

The term "**variable domain**" or "**variable region**" refers to a portion of the light or heavy chains of an antibody that is generally located at the amino-terminal of the light or heavy chain and has a length of about 120 to 130 amino acids in the heavy chain and about 100 to 110 amino acids in the light chain, and are used in the binding and specificity of each particular antibody for its particular antigen. The variable domains differ extensively in sequence between different antibodies. The variability in sequence is concentrated in the CDRs while the less variable portions in the variable domain are referred to as framework regions (FR). Each variable region comprises three CDRs which are connected to four FR. The CDRs of the light and heavy chains are primarily responsible for the interaction of the antibody with antigen. Although not directly involved in antigen binding, the FR determines the folding of the molecules and thus the amount of CDR that is presented on the surface of the variable region for interaction with the antigen. In some embodiments, the variable region is a human variable region.

The "**variable region**" or "**variable domain**" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "V_H." The variable domain of the light chain may be referred to as "V_L." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "**variant**" when used in relation to VISTA or to an anti-VISTA antibody refers to a peptide or polypeptide comprising one or more (such as, for example, about

1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, or about 1 to about 5) amino acid sequence substitutions, deletions, and/or additions as compared to a native or unmodified sequence. For example, a VISTA variant may result from one or more (such as, for example, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, or about 1 to about 5) changes to an amino acid sequence of native VISTA. Also by way of example, a variant of an anti-anti-VISTA antibody may result from one or more (such as, for example, about 1 to about 25, about 1 to about 20, about 1 to about 15, about 1 to about 10, or about 1 to about 5) changes to an amino acid sequence of a native or previously unmodified anti-anti-VISTA antibody. Preferably, a variant of an anti-VISTA antibody may result from one change to an amino acid sequence of a native or previously unmodified anti- anti-VISTA antibody. In some embodiments, the VISTA variant or anti-VISTA antibody variant at least retains VISTA or anti-VISTA antibody functional activity, respectively. In some embodiments, an anti-VISTA antibody variant does not undergo deamidation in the CDRs. In some embodiments, an anti-VISTA antibody variant binds VISTA and/or is antagonistic to VISTA activity. In some embodiments, an anti-VISTA antibody variant does not undergo deamidation in the CDRs, binds VISTA and/or is antagonistic to VISTA activity. Variants may be naturally occurring, such as allelic or splice variants, or may be artificially constructed. In some embodiments, the variant is encoded by a single nucleotide polymorphism (SNP) variant of a nucleic acid molecule that encodes VISTA or anti-VISTA antibody VH or VL regions or subregions. Polypeptide variants may be prepared from the corresponding nucleic acid molecules encoding the variants.

The term "**vector**" refers to a substance that is used to introduce a nucleic acid molecule into a host cell. In particular, a "vector," as used herein, is a nucleic acid molecule capable of propagating another nucleic acid molecule to which it is linked. One example of vector is a "**plasmid**", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another example of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. The term "vector" thus includes the vector as a self-replicating nucleic acid structure as well as the vector

incorporated into the genome of a host cell into which it has been introduced. Vectors applicable for use include, for example, expression vectors, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, which can include selection sequences or markers operable for stable integration into a host cell's chromosome.

Certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "**recombinant expression vectors**" (or simply, "**expression vectors**"). In general, expression vectors of utility in recombinant DNA techniques are in the form of plasmids. In the present specification, "**plasmid**" and "**vector**" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such forms of expression vectors, such as bacterial plasmids, YACs, cosmids, retrovirus, EBV-derived episomes, and all the other vectors that the skilled man will know to be convenient for ensuring the expression of the heavy and/or light chains of the antibody of interest (*e.g.*, an anti-VISTA antibody). The skilled man will realise that the polynucleotides encoding the heavy and the light chains can be cloned into different vectors or in the same vector.

The vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes that can be included, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. When two or more nucleic acid molecules are to be co-expressed (*e.g.* both an antibody heavy and light chain), both nucleic acid molecules can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The introduction of nucleic acid molecules into a host cell can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the nucleic acid molecule is expressed in a sufficient amount to

produce the desired product (*e.g.* an anti-VISTA antibody provided herein), and it is further understood that expression levels can be optimised to obtain sufficient expression using methods well known in the art.

The term "**VISTA**" or "**VISTA polypeptide**" and similar terms refers to the polypeptide ("polypeptide," "peptide" and "protein" are used interchangeably herein) encoded by the human Chromosome 10 Open Reading Frame 54 (VISTA) gene, which is also known in the art as B7-H5, platelet receptor Gi24, GI24, Stress Induced Secreted Protein1, SISP1, and PP2135, for example, comprising the amino acid sequence of:

1 mgvptaleag swrwgslfa lflaaslgpv aafkvatpys lyvcpegqnv tltcrllgpv

61 dkghdvtfyk twyrssrgev qtcserrpir nltfqdlhlh hgghqaants hdlaqrhgle

121 sasdhhgnfs itmrnlttld sglycclvve irhhhsehrv hgamelqvqt gkdapsncvv

181 ypsssqdsen itaaalatga civgilclpl illlvykqrq aasnrraqel vrmdsniqgi

241 enpgfeaspp aqgipeakvr hplsyvaqrq psesgrhlls epstplsppg pgdvffpsld

301 pvpdspnfev i (SEQ ID NO: 1)

and related polypeptides, including SNP variants thereof. The VISTA polypeptide has been shown to or is predicted to comprise several distinct regions within the amino acid sequence including: a signal sequence (residues 1-32; *see* Zhang *et al.*, *Protein Sci.* 13:2819-2824 (2004)); an immunoglobulin domain - IgV-like (residues 33-162); and a transmembrane region (residues 195-215). The mature VISTA protein includes amino acid residues 33-311 of SEQ ID NO: 1. The extracellular domain of the VISTA protein includes amino acid residues 33-194 of SEQ ID NO: 1. Related polypeptides include allelic variants (*e.g.*, SNP variants); splice variants; fragments; derivatives; substitution, deletion, and insertion variants; fusion polypeptides; and interspecies homologs, preferably, which retain VISTA activity and/or are sufficient to generate an anti-VISTA immune response. VISTA can exist in a native or denatured form. The VISTA polypeptides described herein may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods. A "**native sequence VISTA polypeptide**" comprises a polypeptide having the same amino acid sequence as the corresponding VISTA polypeptide derived from nature. Such native sequence VISTA polypeptides can be isolated from nature or can

be produced by recombinant or synthetic means. The term "**native sequence VISTA polypeptide**" specifically encompasses naturally-occurring truncated or secreted forms of the specific VISTA polypeptide *(e.g.,* an extracellular domain sequence), naturally-occurring variant forms *(e.g.,* alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide.

A cDNA nucleic acid sequence encoding the VISTA polypeptide, for example, comprises:

1 atgggcgtcc ccacggccct ggaggccggc agctggcgct ggggatccct gctcttcgct

61 ctcttcctgg ctgcgtccct aggtccggtg gcagccttca aggtcgccac gccgtattcc

121 ctgtatgtct gtcccgaggg gcagaacgtc accctcacct gcaggctctt gggccctgtg

181 gacaaagggc acgatgtgac cttctacaag acgtggtacc gcagctcgag gggcgaggtg

241 cagacctgct cagagcgccg gcccatccgc aacctcacgt tccaggacct tcacctgcac

301 catggaggcc accaggctgc caacaccagc cacgacctgg ctcagcgcca cgggctggag

361 tcggcctccg accaccatgg caacttctcc atcaccatgc gcaacctgac cctgctggat

421 agcggcctct actgctgcct ggtggtggag atcaggcacc accactcgga gcacagggtc

481 catggtgcca tggagctgca ggtgcagaca ggcaaagatg caccatccaa ctgtgtggtg

541 tacccatcct cctcccagga tagtgaaaac atcacggctg cagccctggc tacgggtgcc

601 tgcatcgtag gaatcctctg cctccccctc atcctgctcc tggtctacaa gcaaaggcag

661 gcagcctcca accgccgtgc ccaggagctg gtgcggatgg acagcaacat tcaagggatt

721 gaaaaccccg gctttgaagc ctcaccacct gcccagggga tacccgaggc caaagtcagg

781 caccccctgt cctatgtggc ccagcggcag ccttctgagt ctgggcggca tctgctttcg

841 gagcccagca cccccctgtc tcctccaggc cccggagacg tcttcttccc atccctggac

901 cctgtccctg actctccaaa ctttgaggtc atctag (SEQ ID NO: 2)

VISTA is predominantly expressed on the myeloid cell population, particularly myeloid-derived suppressor cells (MDSCs), neutrophils, monocytes, macrophages, and dendritic cells. VISTA can also be expressed on regulatory T cells and $CD4^+$ naïve T lymphocytes. As described herein, VISTA is an immunomodulator, that is a negative checkpoint regulator of immune responses (*e.g.*, inhibits or suppresses immune responses). VISTA has been identified as a negative checkpoint regulator of T cell function and is known to suppress autoimmune responses in a variety of human and mouse models of autoimmunity. VISTA has in particular been shown to promote tumourigenesis, block T cell function, and modulate the activity of macrophages and immunosuppressive myeloid-derived suppressor cells (MDSCs). VISTA is upregulated on immunosuppressive tumour infiltrating leukocytes such as inhibitory regulatory T cells (Tregs) and MDSCs. The presence of VISTA in the tumour microenvironment hinders effective T cell responses and has been implicated in a number of human cancers including prostate, colon, skin, pancreatic, and lung (ElTanbouly et al. *Clin Exp Immunol*. 200(2):120-130 (2020); Mehta et al. *Sci Rep*. **10**(1):1 5171 (2020); Yuan et al. *Trends Immunol*. **42**(3): 209-227 (2021); Tagliamento et al. *Immunotargets Ther*. **10**: 185-200 (2021); Thakkar et al. *J Immunother Cancer*. **10**(2): e003382 (2022); WO 2015/097536; WO 2016/094837; WO 2017/181139; WO 2019/183040)

Orthologs to the VISTA polypeptide are also well known in the art. For example, the mouse ortholog to the VISTA polypeptide is V-region Immunoglobulin-containing Suppressor of T cell Activation (VISTA) (also known as PD-L3, PD-1H, PD-XL, Pro1412 and UNQ730), which shares approximately 70% sequence identity to the human polypeptide. Orthologs of VISTA can also be found in additional organisms including chimpanzee, cow, rat and zebrafish.

A **"VISTA-expressing cell," "a cell having expression of VISTA"** or a grammatical equivalent thereof refers to a cell that expresses endogenous or transfected VISTA on the cell surface. VISTA expressing cells include VISTA-bearing tumour cells, regulatory T cells (*e.g.*, $CD4^+$ $Foxp3^+$ regulatory T cells), myeloid-derived suppressor cells (*e.g.*, $CD11b^+$ or $CD11b^{high}$ myeloid-derived suppressor cells) and/or suppressive dendritic cells (*e.g.*, $CD11b^+$ or $CD11b^{high}$ dendritic cells). A cell expressing VISTA produces sufficient levels of VISTA on its surface, such that an anti-VISTA antibody can bind thereto and/or PSGL-1 or a cell expressing PSGL-1 can bind thereto. In some aspects, inhibition or blocking of such binding may have a therapeutic effect. A cell that "overexpresses" VISTA is one that has significantly higher levels of VISTA at the cell

surface thereof, compared to a cell of the same tissue type that is known to express VISTA. Such overexpression may be caused by gene amplification or by increased transcription or translation. VISTA overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the VISTA protein present on the surface of a cell (*e.g.* via an immunohistochemistry assay; FACS analysis). Alternatively, or additionally, one may measure levels of VISTA-encoding nucleic acid or mRNA in the cell, *e.g.* via fluorescent in situ hybridisation; (FISH; see W098/45479 published October, 1998), Southern blotting, Northern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). Aside from the above assays, various *in vivo* assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labelled with a detectable agent, and binding of the antibody to cells in the patient can be evaluated, *e.g.* by external scanning for radioactivity or by analysing a biopsy taken from a patient previously exposed to the antibody. A VISTA-expressing tumour cell includes, but is not limited to, acute myeloid leukaemia (AML) tumour cells.

A **"VISTA-mediated disease," "VISTA-mediated disorder"** and **"VISTA-mediated condition"** are used interchangeably and refer to any disease, disorder or condition that is completely or partially caused by or is the result of VISTA. Such diseases, disorders or conditions include those caused by or otherwise associated with VISTA, including by or associated with VISTA-expressing cells (*e.g.*, tumour cells, myeloid-derived suppressor cells (MDSC), suppressive dendritic cells (suppressive DC), and/or regulatory T cells (T-regs)). In some embodiments, VISTA is aberrantly (*e.g.*, highly) expressed on the surface of a cell. In some embodiments, VISTA may be aberrantly upregulated on a particular cell type. In other embodiments, normal, aberrant or excessive cell signalling is caused by binding of VISTA to a VISTA receptor (*e.g.*, PSGL-1, VSIG3, VSIG8, or LRIG1), which can bind or otherwise interact with VISTA. Preferably, a **"VISTA-mediated disease"** as used herein refers to a tumour (*i.e.*, a **"VISTA-mediated tumour"**) whose proliferation is associated with the activity of VISTA. For example, expression of VISTA in cells present in the tumour microenvironment, *e.g.*, MDSCs, may result in suppression of an immune response against the tumour. In a specific instance, expression of VISTA in cells present in the tumour microenvironment, *e.g.*, MDSCs, may result in suppression of T cell immunity ($CD4^+$ and $CD8^+$ T cell immunity) and/or prevention of the expression of

proinflammatory cytokines. Notably, proliferation of $CD4^+$ and or $CD8^+$ T cells may be inhibited. The expression of cytokines such as IFNγ, IL-2, or TNFα, may be prevented. In a specific aspect, these effects are mediated by VISTA expressed on cells present in the tumour microenvironment, *e.g.*, MDSCs, interacting with receptors such as PSG-L1, VSIG3, VSIG8, or LRIG1, which are expressed on immune cells, *e.g.*, T cells, or tumour cells.

Antibody formulations

The present disclosure provides a stable pharmaceutical composition (formulation) comprising an anti-VISTA antibody or an antigen-binding fragment thereof, such as *e.g.*, any of the anti-VISTA antibodies described herein.

These compositions are particularly useful for *e.g.*, stimulating an immune response in a subject. The antibody of the present invention which specifically binds to VISTA induces T cell activation by binding to VISTA protein, which inhibits T cell activation, and thus the antibody can stimulate an immune response.

The compositions described herein are also useful for treating cancer. A protective anti-tumour immunity can be established by administration of such compositions comprising the anti-VISTA antibody, antigen-binding fragments thereof, or conjugates thereof, which are disclosed herein.

Immune checkpoints play crucial roles in maintaining self-tolerance and limiting immune-mediated tissue damage under physiologic conditions. VISTA is a type-I transmembrane protein belonging to the B7-related immunoglobulin superfamily which is highly expressed in the haematopoietic compartment. VISTA acts both as a ligand and a receptor and negatively regulates T-cell activation through inhibiting $CD4^+$ and $CD8^+$ T-cell proliferation and proinflammatory cytokines (e.g., IFNγ, TNFα, or IL-2) production.

A monoclonal antibody Ab3 capable of inhibiting VISTA immune suppression, thereby enhancing antitumour immune response, is described in WO 2016/094837. The CDRS of this antibody are represented by SEQ ID NOS:3-8, and the V_H and V_L by SEQ ID NO:9 and SEQ ID NO:10, respectively. The complete heavy chain of Ab3 has the sequence represented by SEQ ID NO:11 and the complete light chain of Ab3 has the sequence represented by SEQ ID NO:12.

The complete sequence of the antibody Ab3indicates that it contains 11 potential deamidation sites. Whereas all these sites are predicted to be valid deamidation sites, the present inventors have found that only one of them is actually subject to deamidation, *i.e.*, the Asn residue at position in 55 in CDR2 of the heavy chain. Surprisingly, the replacement of this Asn by an Asp residue does not affect the binding of Ab3 to its target, in stark contrast to the teaching of the prior art wherein a similar mutation in a CDR led to a 400-fold decrease of the affinity for the corresponding CD52 antigen (Liu et al., 2022). Moreover, the mutated antibody retains the capacity of inhibiting VISTA immunoinhibitory activity, since it is capable of blocking the interaction between VISTA and each of its two binding partners, PSG-L1 and VSIG3, said interaction resulting in inhibition of T cell function. Accordingly, the mutated antibody inhibits tumour proliferation in vivo.

The present disclosure relates to stable liquid formulations of antibodies or fragments thereof that specifically bind to VISTA, and are not prone to deamidation whilst retaining their capacity of inhibiting VISTA immunoinhibitory activity. In certain embodiments, a stable liquid formulation of an anti-human VISTA antibody or a fragment thereof is suitable for parenteral administration to a human subject. In a specific embodiment, a stable liquid formulation of the disclosure is suitable for subcutaneous administration to a human subject.

The present disclosure relates to sterile, stable aqueous formulations comprising an antibody or fragment thereof that specifically binds human VISTA, is more homogeneous, and is capable of stimulating anti-cancer immunity. The present disclosure provides a formulation of an anti-VISTA antibody described in WO 2022/229469. In a specific instance, a formulation of the disclosure comprises an anti-VISTA antibody comprising an Asp residue at position 55 in CDR2 of the heavy chain. In another embodiment, a formulation of the disclosure comprises an anti-VISTA antibody comprising a heavy chain sequence of SEQ ID NO: 21 and a light chain sequence of SEQ ID NO: 22. In one instance, a formulation of the disclosure is provided in a pre-filled syringe.

In one instance, a liquid formulation of the disclosure is an aqueous formulation. Preferably, a liquid formulation of the disclosure is an aqueous formulation wherein the aqueous carrier is distilled water.

Advantageously, the composition of the disclosure is sterile.

Advantageously, the composition of the disclosure is homogeneous.

Advantageously, the composition of the disclosure is isotonic.

The disclosure encompasses stable liquid formulations comprising a single antibody of interest or antibody fragment thereof, such as, *e.g.*, an antibody that specifically binds to VISTA, notably the antibody described in WO 2022/229469. Optionally, the formulations disclosed herein can comprise one or more additional therapeutic agents, such as the immune checkpoint inhibitors described below.

In one instance, the formulation disclosed herein comprises at least about 1 mg/mL, at least about 5 mg/mL, at least about 10 mg/mL, at least about 15 mg/mL, at least about 20 mg/mL, at least about 30 mg/mL, at least about 40 mg/mL, at least about 50 mg/mL, at least about -60 mg/mL, at least about 70 mg/mL,, at least about 80 mg/mL, at least about 90 mg/mL, or at least about 100 mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469. In another instance, the formulation disclosed herein comprises less than 100 mg/mL, less than 90 mg/mL, less than 80 mg/mL, less than 70 mg/mL, less than 60 mg/mL, less than 50 mg/mL, less than 40 mg/mL, less than 30 mg/mL, less than 20 mg/mL, less than 15 mg/mL, less than 10 mg/mL, or less than 5mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469. In another instance, the formulation disclosed herein comprises between 5 and 100 mg/mL, between 10 and 70 mg/mL, between 15 and 50 mg/mL, or between 20 and 30 mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469. Preferably, the formulation disclosed herein comprises 20 mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469.

Optionally, the formulations of the disclosure may further comprise common excipients and/or additives such as buffering agents, saccharides, salts and surfactants. Additionally or alternatively, the formulations of the disclosure may further comprise common excipients and/or additives, such as, but not limited to, solubilisers, diluents, binders, stabilisers, salts, lipophilic solvents, amino acids, chelators, preservatives, or the like.

In certain instances, the buffering agent is selected from the group consisting of histidine, citrate, phosphate, glycine, and acetate. In other instances, the saccharide excipient is selected from the group consisting of trehalose, sucrose, mannitol, maltose and raffinose. In still other instances, the surfactant is selected from the group consisting of polysorbate 20, polysorbate 40, polysorbate 80, and Pluronic F68. In yet other embodiments the salt is selected from the group consisting of NaCl, KCl, $MgCl_2$, and $CaCl_2$.

The present formulations include a buffering or pH adjusting agent to provide improved pH control, thereby maintaining the pH in the desired range. For example, a formulation as disclosed herein has a pH of between about 3.0 and about 9.0, between about 4.0 and about 8.0, between about 5.0 and about 8.0, between about 5.0 and about 7.0, between about 5.0 and about 6.5, between about 5.5 and about 8.0, between about 5.5 and about 7.0, or between about 5.5 and about 6.5. In a further embodiment, a composition of the disclosure has a pH of about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In a specific embodiment, a composition of the disclosure has a pH of about 6.5.

Suitable buffering agents for use with the present disclosure include both organic and inorganic acids and salts thereof such as citrate buffers (*e.g.*, monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, *etc.*), succinate buffers (*e.g.*, succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, *etc.*), tartrate buffers (*e.g.*, tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, *etc.*), fumarate buffers (*e.g.*, fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, *etc.*), gluconate buffers (*e.g.*, gluconic acid-sodium gluconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, *etc.*), oxalate buffer (*e.g.*, oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, *etc.*), lactate buffers (*e.g.*, lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, *etc.*) and acetate buffers (*e.g.*, acetic acid-sodium acetate

mixture, acetic acid-sodium hydroxide mixture, *etc.*). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used. Preferably, the buffering agent is selected in the group consisting of citrate buffers, phosphate buffers, and histidine buffers. More preferably, the buffering agent is a histidine buffer.

Buffering agents, *e.g.*, histidine, can be present at concentration ranging from about 2 mM to about 50 mM. Preferably, the buffering agent is present at a concentration of at least 2, 5, 10, 15, 20, 25, 30, 35, 40, or 45 mM. Preferably, the buffering agent is present at a concentration of less than 45, 40, 35, 30, 25, 20, 15, 10, 5, or 2 mM. More preferably, the concentration of buffering agent is comprised between 5 and 45 mM, 10 and 40 mM, 15 and 35 mM, 20 and 30 mM. Even more preferably, the concentration of buffering agent is about 25 mM.

Most preferably, the buffering agent is a histidine buffer and is present at a concentration of 25 mM.

The skilled person will understand that the formulations disclosed herein may be isotonic with human blood, that is, the formulations have essentially the same osmotic pressure as human blood. Preferably, the osmotic pressure of the present formulations ranges from about 100 mOSm to about 1200 mOSm, or from about 200 mOSm to about 1000 mOSm, or from about 200 mOSm to about 800 mOSm, or from about 200 mOSm to about 600 mOSm, or from about 250 mOSm to about 500 mOSm, or from about 250 mOSm to about 400 mOSm, or from about 250 mOSm to about 350 mOSm. The present formulations will more preferably have an osmotic pressure from about 250 mOSm to about 350 mOSm. Isotonicity can be measured by, for example, using a vapour pressure or ice-freezing type osmometer. Tonicity of a formulation is adjusted by the use of tonicity modifiers. "Tonicity modifiers" are those pharmaceutically acceptable inert substances that can be added to the formulation to ensure isotonicity of liquid formulations of the present disclosure and include polyhydric sugar alcohols, for example trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol, salts and amino acids.

In certain embodiments, the formulations disclosed herien have an osmotic pressure from about 100 mOSm to about 1200 mOSm, or from about 200 mOSm to about 1000 mOSm, or from about 200 mOSm to about 800 mOSm, or from about 200 mOSm

to about 600 mOSm, or from about 250 mOSm to about 500 mOSm, or from about 250 mOSm to about 400 mOSm, or from about 250 mOSm to about 350 mOSm.

In certain embodiments, the present formulations have an osmotic pressure from 100 mOSm to 1200 mOSm, or from 200 mOSm to 1000 mOSm, or from 200 mOSm to 800 mOSm, or from 200 mOSm to 600 mOSm, or from 250 mOSm to 500 mOSm, or from 250 mOSm to 400 mOSm, or from 250 mOSm to 350 mOSm.

Concentration of any one or any combination of various components of the formulations described herein is adjusted to achieve the desired tonicity of the final formulation. Amino acids that are pharmaceutically acceptable and suitable for this disclosure as tonicity modifiers include, but are not limited to, proline, alanine, L-arginine, asparagine, L-aspartic acid, glycine, serine, lysine, and histidine. The desired isotonicity of the final formulation may notably be achieved by adjusting the salt concentration of the formulations. Salts that are pharmaceutically acceptable and suitable for this disclosure as tonicity modifiers include, but are not limited to, sodium chloride, sodium succinate, sodium sulphate, potassium chloride, magnesium chloride, magnesium sulphate, and calcium chloride. Advantageously, the present formulations comprise NaCl, $MgCl_2$, and/or $CaCl_2$. Preferably, the present formulations comprise NaCl.

In an instance, the tonicity modifier is present in the formulation at a concentration comprised between 0 mM and 300 mM, preferably between 50 mM and 250 mM, more preferably between 100 mM and 200 mM.

In an instance, the formulation disclosed herein comprises at least about 10 mM, at least about 25 mM, at least about 50 mM, at least about 75 mM, at least about 80 mM, at least about 100 mM, at least about 125 mM, at least about 150 mM, at least about 175 mM, at least about 200 mM, or at least about 300 mM NaCl. In an another instance, a formulation described herein comprises between 10 mM and 300 mM, between 10 mM and 200 mM, between 10 mM and 175 mM, between 10 mM and 150 mM, between 25 mM and 300 mM, between 25 mM and 200 mM, between 25 mM and 175 mM, between 25 mM and 150 mM, between 50 mM and 300 mM, between 50 mM and 200 mM, between 50 mM and 175 mM, between 50 mM and 150 mM, between 75 mM and 300 mM, between 75 mM and 200 mM, between 75 mM and 175 mM, between 75 mM and 150 mM, between 100 mM and 300 mM, between 100 mM and 200 mM, between 100 mM and 175 mM, or between 100 mM and 150 mM NaCl. In yet another

instance, the formulation disclosed herein comprises between 0 mM and 300 mM, between 50 mM and 250 mM, or between 100 mM and 200 mM NaCl. Preferably, a formulation of the disclosure comprises 150 mM NaCl.

Preservatives can be added to retard microbial growth, and can be added in amounts ranging from 0.2%-1% (w/v). Suitable preservatives for use with the present disclosure include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (*e.g.*, chloride, bromide, and iodide), hexamethonium chloride, and alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Stabilisers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilises the therapeutic agent (*i.e.*, an anti-VISTA antibody, an antigen-binding fragment thereof, or a conjugate thereof) or helps to prevent denaturation or adherence to the container wall. Typical stabilisers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, *etc.*, organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (*e.g.*, peptides of 10 residues or fewer); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophylic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccacharides such as raffinose; and polysaccharides such as dextran. Stabilisers can be present in the range from 0.1 to 10,000 weights per part of weight active protein (*e.g.*, an anti-VISTA antibody or antigen-binding fragment thereof comprising such an antibody). Preferably, the formulation described herein comprises at least one stabiliser selected from arginine and sucrose. Arginine may for example be present at a concentration comprised between 0 and 50 mM. In another instance, the concentration sucrose may range from 0 to 6 %.

Non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilise the anti-VISTA antibody as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to

be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80, *etc.*), polyoxamers (184, 188, *etc.*), pluronic polyols, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, *etc.*). Non-ionic surfactants can be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, for example about 0.07 mg/ml to about 0.2 mg/ml. Preferably, the formulation described herein comprises a non-ionic surfactant which is a polysorbate, such as *e.g.*, Polysorbate 20 or Polysorbate 80. Preferably, the non-ionic surfactant in the formulation disclosed herein is polysorbate 80. In one instance, the formulation described herein comprises at least about 0.001%, at least about 0.002%, at least about 0.003%, at least about 0.004%, at least about 0.005%, at least about 0.006%, at least about 0.007%, at least about 0.008%, at least about 0.009%, at least about 0.01%, at least about 0.02%, at least about 0.05%, at least about 0.1%, at least about 0.2%, or at least about 0.5% Polysorbate 80 (v/v). In another instance, the formulation described herein comprises between about 0.001% and about 0.5% (v/v), between about 0.002% and about 0.2% (v/v), between about 0.003% and about 0.1% (v/v), between about 0.004% and about 0.009% (v/v), between about 0.005% and about 0.008% (v/v), or between about 0.006% and about 0.007% Polysorbate 80 (v/v). In a further instance, the formulation disclosed herein comprises about 0.006% Polysorbate 80 (v/v).

Additional miscellaneous excipients include bulking agents (*e.g.*, starch), chelating agents (*e.g.*, EDTA), antioxidants (*e.g.*, ascorbic acid, methionine, vitamin E), and cosolvents.

Preferably, the formulations disclosed herein comprise histidine buffer, NaCl, Polysorbate 80 and the anti-VISTA antibody disclosed in WO 2022/229469, or a fragment thereof. Optionally, the formulations disclosed herein comprise sucrose.

Advantageously, the formulations disclosed herein comprise between 10mM and 50mM Histidine, preferably 25mM; between 0 and 150mM NaCl, preferably 150mM; between 0 and 0.5 % polysorbate 80 (v/v), preferably 0.006% (v/v); and between 0 and 6% sucrose, preferably 0%; at a pH comprised between 5.5 and 7.0, preferably 6.5.

Preferably, the formulations disclosed herein comprises 25 mM Histidine, 150 mM NaCl, 0.006% Polysorbate 80 (v/v), pH 6.5. More preferably, this pharmaceutical composition comprises 20 mg/mL of the anti-VISTA antibody disclosed in WO 2022/229469, or a fragment thereof.

The formulation will usually be non-pyrogenic e.g., containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The formulation is preferably gluten-free.

The anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, may be admixed with a second therapeutic agent in the present formulations, as described below.

The formulation will usually be supplied as part of a sterile, pharmaceutical composition that will normally include a pharmaceutically acceptable carrier and/or excipient. In another aspect, the disclosure thus provides a pharmaceutical composition comprising the anti-VISTA antibody or antigen-binding fragment thereof, and a pharmaceutically acceptable carrier and/or an excipient.

This composition can be in any suitable form (depending upon the desired method of administering it to a patient). The compositions utilised in the methods described herein can be administered, for example, intravitreally (*e.g.*, by intravitreal injection), by eye drop, intramuscularly, intravenously, intradermally, percutaneously, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intrathecally, intranasally, intravaginally, intrarectally, topically, intratumourally, peritoneally, subcutaneously, subconjunctivally, intravesicularly, mucosally, intrapericardially, intraumbilically, intraocularly, intraorbitally, orally, topically, transdermally, by inhalation, by injection, by implantation, by infusion, by continuous infusion, by localised perfusion bathing target cells directly, by catheter, by lavage, in cremes, or in lipid compositions. The compositions utilised in the methods described herein can also be administered systemically or locally. The method of administration can vary depending on various factors (*e.g.*, the compound or composition being administered and the severity of the condition, disease, or disorder being treated). The most suitable route for administration in any given case will depend on the particular antibody, the subject, and the nature and severity of the disease and the physical condition of the subject. For example, the anti-VISTA antibody or an antigen-binding fragment thereof can be formulated as an aqueous solution and administered by subcutaneous injection. Preferably, the anti-VISTA is formulated as an aqueous solution and administered by infusion.

The present formulations can be conveniently presented in unit dose forms containing a predetermined amount of an anti-VISTA or an antigen-binding fragment thereof per dose. Such a unit can contain for example but without limitation 5 mg to 5 g, for example 10 mg to 1 g, or 20 to 50 mg. Pharmaceutically acceptable carriers for use in the disclosure can take a wide variety of forms depending, *e.g.*, on the condition to be treated or route of administration.

The formulations disclosed herein can be prepared for storage as lyophilised formulations or aqueous solutions by mixing the antibody having the desired degree of purity with optional pharmaceutically-acceptable carriers, excipients or stabilisers typically employed in the art (all of which are referred to herein as "carriers"), *e.g.*, buffering agents, stabilising agents, preservatives, isotonifiers, non-ionic detergents, antioxidants, and other miscellaneous additives. *See*, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be nontoxic to the recipients at the dosages and concentrations employed. Preferably, the formulation disclosed herein is a liquid formulation. More preferably, the liquid formulation of the disclosure is an aqueous composition. Still more preferably, the liquid formulation disclosed herein is an aqueous composition wherein the aqueous carrier is distilled water.

Stability of Formulations

In an aspect, the formulation disclosed herein stabilises an anti-VISTA antibody or a fragment thereof. Preferably, the formulation disclosed herein prevents aggregation of an anti-VISTA antibody or fragment thereof. In a specific instance, a formulation of the disclosure comprises the anti-VISTA antibody comprising a heavy chain sequence of SEQ ID NO:21 and a light chain sequence of SEQ ID NO:22, e.g., the anti-VISTA antibody disclosed in WO 2022/229469, or a fragment thereof.

The present disclosures provide stable liquid formulations comprising the anti-VISTA antibodies disclosed herein. The stability of said antibody can be assessed by degrees of aggregation, degradation or fragmentation, as measured by HPSEC, reverse phase chromatography, static light scattering (SLS), Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), urea unfolding techniques, intrinsic tryptophan fluorescence, differential scanning calorimetry, and/or ANS binding techniques, compared to a reference formulation comprising a reference antibody. The overall stability of a formulation comprising an antibody

(including antibody fragment thereof) may also be assessed by various immunological assays including, for example, ELISA and radioimmunoassay using isolated antigen molecules. Furthermore, the stability of a formulation comprising an antibody may also be assessed using various assays designed to measure a functional characteristic of the antibody, for example, assays designed to measure antigen binding affinity, in vitro ADCC activity, in vivo depletion activity, in vitro CDC activity.

Preferably, the formulation disclosed herein is stable upon storage at about 5°C for at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, at least about 6 months, at least 12 months, at least 18 months, at least 24 months, at least 30 months, at least 36 months, at least 42 months, or at least 48 months. More preferably, the formulation disclosed herein is stable upon storage at about 5°C for at least about 1 year, at least about 2 years, at least about 3 years, at least about 4 years, at least about 5 years, at least about 6 years, at least about 7 years, at least about 8 years, at least about 9 years, at least about 10 years, at least about 11 years, or at least about 12 years.

In one embodiment, the formulation disclosed herein comprises an anti-VISTA antibody that has a VISTA binding activity that is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the VISTA binding activity of a reference antibody, wherein the formulation was stored at about 5°C for at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, at least about 6 months, at least 12 months, at least 18 months, at least 24 months, at least 30 months, at least 36 months, at least 42 months, or at least 48 months. More preferably, the formulation disclosed herein comprises an anti-VISTA antibody that has a VISTA binding activity that is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the VISTA binding activity of a reference antibody, wherein the formulation was stored at about 5°C for at least about 1 year, at least about 2 years, at least about 3 years, at least about 4 years, at least about 5 years, at least about 6 years, at least about 7 years, at least about 8 years, at least about 9 years, at least about 10 years, at least about 11 years, or at least about 12 years.

In one embodiment, the formulation disclosed herein comprises an anti-VISTA antibody, wherein less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 7% or less than 10% of the antibody forms an aggregate as determined by SEC

upon storage at about 5°C for at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, at least about 6 months, at least 12 months, at least 18 months, at least 24 months, at least 30 months, at least 36 months, at least 42 months, or at least 48 months. More preferably, the formulation disclosed herein comprises an anti-VISTA antibody, wherein less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 7% or less than 10% of the antibody forms an aggregate as determined by SEC upon storage at about 5°C for at least about 1 year, at least about 2 years, at least about 3 years, at least about 4 years, at least about 5 years, at least about 6 years, at least about 7 years, at least about 8 years, at least about 9 years, at least about 10 years, at least about 11 years, or at least about 12 years.

In one embodiment, the formulation disclosed herein comprises an anti-VISTA antibody, wherein less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 7% or less than 10% of the antibody is fragmented as determined by SEC upon storage at about 5°C for at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, at least about 6 months, at least 12 months, at least 18 months, at least 24 months, at least 30 months, at least 36 months, at least 42 months, or at least 48 months. More preferably, the formulation disclosed herein comprises an anti-VISTA antibody, wherein less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 7% or less than 10% of the antibody is fragmented as determined by SEC upon storage at about 5°C for at least about 1 year, at least about 2 years, at least about 3 years, at least about 4 years, at least about 5 years, at least about 6 years, at least about 7 years, at least about 8 years, at least about 9 years, at least about 10 years, at least about 11 years, or at least about 12 years.

In one embodiment, the formulation disclosed herein comprises an anti-VISTA antibody, wherein less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 7% or less than 10% of the antibody is not a full-length antibody (H2L2) as determined by CE-SDS upon storage at about 5°C for at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, at least about 6 months, at least 12 months, at least 18 months, at least 24 months, at least 30 months, at least 36 months, at least 42 months, or at least 48 months. More preferably, the formulation disclosed herein comprises an anti-VISTA antibody, wherein less than 1%, less than 2%, less than 3%, less than 4%, less than 5%,

less than 7% or less than 10% the antibody is not a full-length antibody (H2L2) as determined by CE-SDS upon storage at about 5°C for at least about 1 year, at least about 2 years, at least about 3 years, at least about 4 years, at least about 5 years, at least about 6 years, at least about 7 years, at least about 8 years, at least about 9 years, at least about 10 years, at least about 11 years, or at least about 12 years.

Formulations with high concentration of antibody may have short shelf lives and the formulated antibodies may lose biological activity resulting from chemical and physical instabilities during the storage. Among those, aggregation, deamidation and oxidation are known to be the most common causes of antibody degradation. In particular, aggregation can potentially lead to increased immune response in patients, leading to safety concerns. Thus it must be minimised or prevented.

Preferably, the formulations of the disclosure maintain improved aggregation profiles upon storage, for example, for extended periods (for example, but not limited to 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 3 years, 4 years, or 5 years) at 5°C.

Numerous methods useful for determining the degree of aggregation, and/or types and/or sizes of aggregates present in a protein formulation (e.g., antibody formulation of the disclosure) are known in the art, including but not limited to, size exclusion chromatography (SEC), high performance size exclusion chromatography (HPSEC), static light scattering (SLS), Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), urea-induced protein unfolding techniques, intrinsic tryptophan fluorescence, differential scanning calorimetry, and 1-anilino-8-naphthalenesulfonic acid (ANS) protein binding techniques. For example, size exclusion chromatography (SEC) may be performed to separate molecules on the basis of their size, by passing the molecules over a column packed with the appropriate resin, the larger molecules (*e.g.*, aggregates) will elute before smaller molecules (*e.g.*, monomers). The molecules are generally detected by UV absorbance at 280 nm and may be collected for further characterisation. High pressure liquid chromatographic columns are often utilised for SEC analysis (HP-SEC). Specific SEC methods are detailed in the "Examples" presented herein. Alternatively, analytical ultracentrifugation (AUC) may be utilised. AUC is an orthogonal technique which determines the sedimentation coefficients (reported in Svedberg, S) of macromolecules in a liquid sample. Like SEC, AUC is capable of separating and detecting antibody

fragments/aggregates from monomers and is further able to provide information on molecular mass. Protein aggregation in the formulations may also be characterised by particle counter analysis using a coulter counter or by turbidity measurements using a turbidimeter. Turbidity is a measure of the amount by which the particles in a solution scatter light and, thus, may be used as a general indicator of protein aggregation. In addition, non-reducing polyacrylamide gel electrophoresis (PAGE) or capillary gel electrophoresis (CGE) may be used to characterise the aggregation and/or fragmentation state of antibodies or a fragment thereof in a formulation of the disclosure.

Anti-VISTA antibodies used in the formulations

Immune checkpoints play crucial roles in maintaining self-tolerance and limiting immune-mediated tissue damage under physiologic conditions. VISTA is a type-I transmembrane protein belonging to the B7-related immunoglobulin superfamily which is highly expressed in the haematopoietic compartment. VISTA acts both as a ligand and a receptor and negatively regulates T-cell activation through inhibiting $CD4^+$ and $CD8^+$ T-cell proliferation and proinflammatory cytokines (e.g., IFNγ, TNFα, or IL-2) production.

A monoclonal antibody Ab3 capable of inhibiting VISTA immune suppression, thereby enhancing antitumour immune response, is described in WO 2016/094837. The CDRS of this antibody are represented by SEQ ID NOS:3-8, and the V_H and V_L by SEQ ID NO:9 and SEQ ID NO:10, respectively. The complete heavy chain of Ab3 has the sequence represented by SEQ ID NO:11 and the complete light chain of Ab3 has the sequence represented by SEQ ID NO:12.

The complete sequence of the antibody Ab3 indicates that it contains 11 potential deamidation sites. Whereas all these sites are predicted to be valid deamidation sites, the present inventors have found that only one of them is actually subject to deamidation, *i.e.*, the Asn residue at position in 55 in CDR2 of the heavy chain. Surprisingly, the replacement of this Asn by an Asp residue does not affect the binding of Ab3 to its target, in stark contrast to the teaching of the prior art wherein a similar mutation in a CDR led to a 400-fold decrease of the affinity for the corresponding CD52 antigen (Liu et al., 2022). Moreover, the mutated antibody retains the capacity of inhibiting VISTA immunoinhibitory activity, since it is capable of blocking the interaction between VISTA and each of its two binding partners, PSG-L1 and VSIG3,

said interaction resulting in inhibition of T cell function. Accordingly, the mutated antibody inhibits tumour proliferation in vivo.

In a first aspect, the present disclosure provides a novel anti-VISTA antibody wherein the antibody comprises a substitution of an Asp for an Asn in the CDR2 of the heavy chain.

Anti-VISTA monoclonal antibodies as used herein include, but are not limited to, synthetic antibodies, recombinantly produced antibodies, multispecific antibodies (including bi-specific antibodies), human antibodies, humanised antibodies, camelised antibodies, chimeric antibodies, intrabodies, anti-idiotypic (anti-Id) antibodies, and functional fragments of any of the above. Anti-VISTA monoclonal antibodies can be of human or non-human origin. Examples of anti-VISTA antibodies of non-human origin include but are not limited to, those of mammalian origin (*e.g.*, simians, rodents, goats, and rabbits). Because every structure of the human antibody originates from a human, there is only low probability of having an immune response compared to a conventional humanised antibody or mouse antibody, and thus it has an advantage that it does not cause any undesirable immune response when administered to a human. Therefore, it can be very advantageously used as an antibody for treatment. Accordingly, anti-VISTA monoclonal antibodies for therapeutic use in humans are preferably humanised or fully human. More preferably, they are humanised.

The antibody disclosed herein is an antibody with substantially the same affinity to the antigen as the antibody Ab3. The term "affinity" indicates a property of specifically recognising and binding to a specific antigen site, and, together with specificity of an antibody for an antigen, the high affinity is an important factor in an immune reaction. In the present case, affinity of the presently disclosed antibodies may be determined by competitive ELISA. Other than this method, various methods for measuring the affinity for an antigen may be employed, and the surface plasmon resonance technology is one example of those methods.

Within a range in which VISTA can be specifically recognised, the monoclonal antibody disclosed herein may include not only the sequence of anti-VISTA antibody of the present invention, which is described in the present specification, but also a biological equivalent thereof, wherein the biological equivalent displays improved binding affinity and/or other biological characteristics of an antibody. For example,

to have further improvement of the binding affinity and/or other biological characteristics of an antibody, additional changes can be made on the amino acid sequence of an antibody. Included in those modifications are deletion, insertion, and/or substitution of the amino acid sequence of an antibody, for example. Those modifications of an amino acid are made based on relative similarity among side-chain substituents of an amino acid, for example, hydrophobicity, hydrophilicity, charge, size, or the like. Based on the analysis of the size, shape, and type of the side-chain substituents of an amino acid, it is found that all of arginine, lysine, and histidine are a residue with positive charge; alanine, glycine, and serine have a similar size; and phenylalanine, tryptophan, and tyrosine have a similar shape. Accordingly, it can be said based on those considerations that, biologically, arginine, lysine, and histidine; alanine, glycine, and serine; and phenylalanine, tryptophan, and tyrosine are functional equivalents.

In an embodiment, the anti-VISTA monoclonal antibodies described herein can be in the form of full-length antibodies, multiple chain or single chain antibodies, fragments of such antibodies that selectively bind to VISTA (including but not limited to Fab, Fab', $(Fab')_2$, Fv, and scFv), surrobodies (including surrogate light chain construct), single domain antibodies, humanised antibodies, camelised antibodies and the like. They also can be of, or derived from, any isotype, including, for example, IgA (*e.g.*, Ig1l or IgA2), IgD, IgE, IgG (*e.g.*, IgG1, IgG2, IgG3 or IgG4), or IgM. In some embodiments, the anti-VISTA antibody is an IgG (*e.g.*, IgG1, IgG2, IgG3 or IgG4). In an embodiment, the antibody further comprises a human constant region. In a further embodiment, the human constant region is selected from the group consisting of IgG1, IgG2, IgG2, IgG3and IgG4. In a still further specific embodiment, the human constant region is IgG1. Furthermore, the heavy chain constant region has gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ε) types, and, as a subclass, it has gamma1 (γ1), gamma2 (γ2), gamma3 (γ3), gamma4 (γ4), alpha1 (α1) and alpha2 (α2). The light chain constant region has kappa (κ) and lambda (λ) types.

In the context of the present disclosure, antibodies comprising a human IgG1 constant region are particularly preferred. Not only do they bind to VISTA with the same affinity as the antibody Ab3, they are also capable of inhibiting the VISTA immunosuppressive effect. Surprisingly, this activity requires the effector functions of the antibodies, which had never been documented for the anti-VISTA antibody Ab3.

Preferably, the anti-VISTA antibody disclosed herein comprises a heavy chain of sequence SEQ ID NO:21 and a light chain of sequence SEQ ID NO:22.

This antibody is more stable and more homogeneous than the antibody Ab3, since it comprises an Asp at position 55 and is thus not subject to deamidation. In addition, this antibody has the same affinity as the antibody Ab3 and inhibits VISTA immune suppressive activity. This inhibition is, in particular, the result of the disruption of the interaction between VISTA and each of its binding partners, PSG-L1 and VSIG-3. In contrast, there is no indication that the antibody Ab3 interferes with these interactions. Moreover, the inhibition of VISTA immune suppression surprisingly requires the effector functions of the antibody disclosed herein.

The sequences of Ab1 and Ab3 are set out in Table 2 below:

Table 2

Ab	SEQ ID NO:	Sequences
Ab3	CDR-H1	3	GFSFTGYT
	CDR-H2	4	ISPYNGGT
	CDR-H3	5	ARRAYGYAMDY
	CDR-L1	6	SSVSY
	CDR-L2	7	DTS
	CDR-L3	8	QQWSSYPFT
	VH	9	EVQLQQSGPELVKPGASMKISCKASGFSFTGYTMNWVKQSHVKNLEWIG LISPYNGGTSYNQKFKGKATLTVDKSSSTAYMELLSLTSEDSAVYYCARRA YGYAMDYWGQGTSVTVSS
	VL	10	QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMYWYQQKPGSSPRLLIYDTS NLASGVPLRFSGSGSGTSYSLTISRMEAEDAATYYCQQWSSYPFTFGSGTK LEIK
	CH	11	QVQLVQSGAEVKKPGASVKISCKASGFSFTGYTMNWVRQAPGQGLEWIG LISPYNGGTSYAQKFQGRATLTVDTSTSTAYMELSSLRSEDTAVYYCARRA YGYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKD TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
	CL	12	EIVLTQSPATLSLSPGERVTMSCSASSSVSYMYWYQQKPGQAPRLLIYDTS NLASGVPARFSGSGSGTDYTLTISSMEPEDFAVYYCQQWSSYPFTFGQGT KLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA LQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC
Ab1	CDR-H1	13	GFSFTGYT
	CDR-H2	14	ISPYDGGT
	CDR-H3	15	ARRAYGYAMDY
	CDR-L1	16	SSVSY

CDR-L2	17	DTS
CDR-L3	18	QQWSSYPFT
VH	19	EVQLQQSGPELVKPGASMKISCKASGFSFTGYTMNWVKQSHVKNLEWIG LISPYDGGTSYNQKFKGKATLTVDKSSSTAYMELLSLTSEDSAVYYCARRA YGYAMDYWGQGTSVTVSS
VL	20	QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMYWYQQKPGSSPRLLIYDTS NLASGVPLRFSGSGSGTSYSLTISRMEAEDAATYYCQQWSSYPFTFGSGTK LEIK
CH	21	QVQLVQSGAEVKKPGASVKISCKASGFSFTGYTMNWVRQAPGQGLEWIG LISPYDGGTSYAQKFQGRATLTVDTSTSTAYMELSSLRSEDTAVYYCARRA YGYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI CNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKD TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
CL	22	EIVLTQSPATLSLSPGERVTMSCSASSSVSYMYWYQQKPGQAPRLLIYDTS NLASGVPARFSGSGSGTDYTLTISSMEPEDFAVYYCQQWSSYPFTFGQGT KLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA LQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP VTKSFNRGEC

Preferably, the antibody of the present formulation is the anti-VISTA antibody disclosed in WO 2022/229469, or a fragment thereof.

Nucleic acids and expression systems

The present disclosure encompasses polynucleotides encoding immunoglobulin light and heavy chain genes for antibodies, notably anti-VISTA antibodies, vectors comprising such nucleic acids, and host cells capable of producing the antibodies of the disclosure. Also provided herein are polynucleotides that hybridise under high stringency, intermediate or lower stringency hybridisation conditions, *e.g.*, as defined supra, to polynucleotides that encode an antibody or modified antibody provided herein.

In a first aspect, the present disclosure relates to one or more polynucleotides encoding an antibody, notably an antibody capable of binding specifically to VISTA, or a fragment thereof, as described above. The present disclosure notably provides a polynucleotide encoding the heavy chain and/or the light chain of the anti-VISTA antibody disclosed herein. More specifically, in certain embodiments, nucleic acid molecules provided herein comprise or consist of a nucleic acid sequence encoding the heavy chain variable region and light chain variable region disclosed herein, or any combination thereof (*e.g.*, as a nucleotide sequence encoding an antibody provided

herein, such as *e.g.*, a full-length antibody, heavy and/or light chain of an antibody, or a single chain antibody provided herein).

For example, the polynucleotide encodes three heavy-chain CDRs of the anti-VISTA antibody described herein. For example, the polynucleotide encodes three light-chain CDRs of the anti-VISTA antibody described herein. For example, the polynucleotide encodes three heavy-chain CDRs and three light-chain CDRs of the anti-VISTA antibody described herein. Another example provides a couple of polynucleotides, wherein the first polynucleotide encodes three heavy-chain CDRs of the anti-VISTA antibody described herein; and the second polynucleotide encodes three light-chain CDRs of the same anti-VISTA antibody described herein.

In another instance, the polynucleotide encodes the heavy-chain variable region of the anti-VISTA antibody described herein. For instance, the polynucleotide encodes the light-chain variable region of the anti-VISTA antibody described herein. For instance, the polynucleotide encodes the heavy-chain variable region and the light-chain variable region of the anti-VISTA antibody described herein. Another instance provides a couple of polynucleotides, wherein the first polynucleotide encodes the heavy-chain variable region of the anti-VISTA antibody described herein; and the second polynucleotide encodes the light-chain variable region of the same anti-VISTA antibody described herein.

In an embodiment, the polynucleotide encodes the heavy-chain of the anti-VISTA antibody described herein. In an embodiment, the polynucleotide encodes the light-chain of the anti-VISTA antibody described herein. In an embodiment, the polynucleotide encodes the heavy-chain and the light-chain of the anti-VISTA antibody described herein. Another embodiment provides a couple of polynucleotides, wherein the first polynucleotide encodes the heavy-chain of the anti-VISTA antibody described herein; and the second polynucleotide encodes the light-chain of the same anti-VISTA antibody described herein.

In an embodiment, the polynucleotide encodes the heavy chain of the anti-VISTA antibody described above is provided. Preferably, the heavy chain comprises three heavy-chain CDRs of sequence SEQ ID NOS: 13-15. More preferably, the heavy chain comprises a heavy chain comprising the variable region of sequence SEQ ID NO:19. Even more preferably, the heavy chain has the sequence represented by SEQ ID NO:21.

In another embodiment, the polynucleotide encodes the light chain of an anti-VISTA antibody described above. Preferably, said light chain comprises three light-chain CDRs of sequence SEQ ID NOS:16-18. More preferably, said light chain comprises a light chain comprising the variable region of sequence SEQ ID NO:20. Even more preferably, the light chain has the sequence represented by SEQ ID NO:222.

Due to the codon degeneracy or in consideration of a codon preferred in an organism in which the light chain and heavy chain of human antibody or a fragment thereof is to be expressed, the polynucleotide encoding the light chain and heavy chain of the monoclonal antibody of the present invention or an antigen-binding fragment thereof can have various variations in the coding region within a range in which the amino acid sequence of the light chain and heavy chain of an antibody expressed from the coding region is not changed, and, even in a region other than the coding region, various changes or modifications can be made within a range in which the gene expression is not affected by them. The skilled person will easily understand that those variant genes also fall within the scope of the present invention. Namely, as long as a protein having the equivalent activity is encoded by the polynucleotide of the present invention, one or more nucleic acid bases can be changed by substitution, deletion, insertion, or a combination thereof, and those also fall within the scope of the present invention. Sequence of the polynucleotide may be either a single chain or a double chain, and it may be either a DNA molecule or an RNA (mRNA) molecule.

According to the invention, a variety of expression systems may be used to express the antibody of the invention. In one aspect, such expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transiently transfected with the appropriate nucleotide coding sequences, express an IgG antibody in situ.

The disclosure provides vectors comprising the polynucleotides described above. In one embodiment, the vector contains a polynucleotide encoding a heavy chain of the anti-VISTA antibody of interest. In another embodiment, the polynucleotide encodes the light chain of the anti-VISTA antibody of interest. In another embodiment, the polynucleotide encodes the heavy chain and the light chain of the anti-VISTA antibody of interest. In yet another embodiment, a couple of polynucleotides are provided, wherein the first polynucleotide encodes the heavy

chain of the anti-VISTA antibody of interest, and the second polynucleotide encodes the light chain of the same anti-VISTA antibody of interest.

The disclosure also provides vectors comprising polynucleotide molecules encoding fusion proteins, modified antibodies, antibody fragments, and probes thereof.

In order to express the heavy and/or light chain of the anti-VISTA antibody of interest, the polynucleotides encoding said heavy and/or light chains are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational sequences. In a preferred embodiment, these polynucleotides are cloned into two vectors.

"Operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. The term "expression control sequence" as used herein refers to polynucleotide sequences which are necessary to affect the expression and processing of coding sequences to which they are ligated. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilise cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

Polynucleotides of the invention and vectors comprising these molecules can be used for the transformation of a suitable host cell. The term "host cell", as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced in order to express the anti-VISTA antibody of interest. It should be understood that such terms are intended to refer not only to the particular subject

cell but also to 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, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

Transformation can be performed by any known method for introducing polynucleotides into a cell host. Such methods are well known of the man skilled in the art and include dextran-mediated transformation, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide into liposomes, biolistic injection and direct microinjection of DNA into nuclei.

The host cell may be co-transfected with one or more expression vectors. For example, a host cell can be transfected with a vector encoding both the heavy chain and the light chain of the anti-VISTA antibody of interest, as described above. Alternatively, the host cell can be transformed with a first vector encoding the heavy chain of the anti-VISTA antibody of interest, and with a second vector encoding the light chain of said antibody. Mammalian cells are commonly used for the expression of a recombinant therapeutic immunoglobulins, especially for the expression of whole recombinant antibodies. For example, mammalian cells such as HEK293 or CHO cells, in conjunction with a vector, containing the expression signal such as one carrying the major intermediate early gene promoter element from human cytomegalovirus, are an effective system for expressing the humanised anti-VISTA antibody of the invention (Foecking et al., 1986, *Gene* **45**:101; Cockett et al., 1990, *Bio/Technology* **8**: 2).

In addition, a host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing of protein products may be important for the function of the protein. Different host cells have features and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems are chosen to ensure the correct modification and processing of the expressed antibody of interest. Hence, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, COS, HEK293, NS/0, BHK, Y2/0, 3T3 or myeloma cells (all these cell lines are available from public depositories such as the

Collection Nationale des Cultures de Microorganismes, Paris, France, or the American Type Culture Collection, Manassas, VA, U.S.A.).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. In one embodiment of the invention, cell lines which stably express the antibody may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells are transformed with DNA under the control of the appropriate expression regulatory elements, including promoters, enhancers, transcription terminators, polyadenylation sites, and other appropriate sequences known to the person skilled in art, and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for one to two days in an enriched media, and then are moved to a selective media. The selectable marker on the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into a chromosome and be expanded into a cell line. Other methods for constructing stable cell lines are known in the art. In particular, methods for site-specific integration have been developed. According to these methods, the transformed DNA under the control of the appropriate expression regulatory elements, including promoters, enhancers, transcription terminators, polyadenylation sites, and other appropriate sequences is integrated in the host cell genome at a specific target site which has previously been cleaved (Moele et al., *Proc. Natl. Acad. Sci. U.S.A.*, **104**(9): 3055-3060; US 5,792,632; US 5,830,729; US 6,238,924; WO 2009/054985; WO 03/025183; WO 2004/067753).

A number of selection systems may be used according to the invention, including but not limited to the Herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al., Proc Natl Acad Sci USA 48: 202, 1992), glutamate synthase selection in the presence of methionine sulfoximide (Adv Drug Del Rev, 58: 671, 2006, and website or litreature of Lonza Group Ltd.) and adenine phosphoribosyltransferase (Lowy et al., Cell 22: 817, 1980) genes in tk, hgprt or aprt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., Proc Natl Acad Sci USA 77: 357, 1980); gpt, which confers resistance to mycophenolic acid (Mulligan et al., Proc Natl Acad Sci USA 78: 2072, 1981); neo, which confers resistance to the aminoglycoside, G-418 (Wu et al., Biotherapy 3: 87, 1991); and hygro, which confers resistance to hygromycin (Santerre et al., Gene 30: 147, 1984). Methods known in the art of recombinant DNA

technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley & Sons (1993). The expression levels of an antibody can be increased by vector amplification. When a marker in the vector system expressing an antibody is amplifiable, an increase in the level of inhibitor present in the culture will increase the number of copies of the marker gene. Since the amplified region is associated with the gene encoding the IgG antibody of the invention, production of said antibody will also increase (Crouse et al., *Mol Cell Biol* **3**: 257, 1983). Alternative methods of expressing the gene of the invention exist and are known to the person of skills in the art. For example, a modified zinc finger protein can be engineered that is capable of binding the expression regulatory elements upstream of the gene of the invention; expression of the said engineered zinc finger protein (ZFN) in the host cell of the invention leads to increases in protein production (see *e.g.* Reik et al., *Biotechnol. Bioeng.*, **97**(5): 1180-1189, 2006). Moreover, ZFN can stimulate the integration of a DNA into a predetermined genomic location, resulting in high-efficiency site-specific gene addition (Moehle et al, *Proc Natl Acad Sci USA*, **104**: 3055, 2007).

The anti-VISTA antibody of interest may be prepared by growing a culture of the transformed host cells under culture conditions necessary to express the desired antibody. The resulting expressed antibody may then be purified from the culture medium or cell extracts. Soluble forms of the anti-VISTA antibody of interest can be recovered from the culture supernatant. It may then be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly by Protein A affinity for Fc, and so on), centrifugation, differential solubility or by any other standard technique for the purification of proteins. Suitable methods of purification will be apparent to a person of ordinary skills in the art.

Another aspect of the invention thus relates to a method for the production of an antibody (*e.g.*, an anti-VISTA antibody) described herein, said method comprising the steps of:

a) growing the above-described host cell in a culture medium under suitable culture conditions; and

b) recovering the antibody (*e.g.*, an anti-VISTA antibody), from the culture medium or from said cultured cells.

The antibody obtained by culturing the transformant can be used in a non-purified state. Impurities can be removed by additional various commons methods like centrifuge or ultrafiltration, and the resultant may be subjected to dialysis, salt precipitation, chromatography or the like, in which the method may be used either singly or in combination thereof. Among them, affinity chromatography is most widely used, including ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, and the like.

Effective amounts

The anti-VISTA antibodies and conjugates thereof, optionally in combination with immune checkpoint inhibitors, will generally be used in an amount effective to achieve the intended result, for example an amount effective to treat cancer in a subject in need thereof. Pharmaceutical compositions comprising anti-VISTA antibodies (or conjugates thereof) and/or immune checkpoint inhibitors can be administered to patients (*e.g.*, human subjects) at therapeutically effective dosages.

Determination of the effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. Toxicity and therapeutic efficacy of a compound or a conjugate can be determined by standard pharmaceutical procedures in cell cultures and in experimental animals. The effective amount of present combination or other therapeutic agent to be administered to a subject will depend on the stage, category and status of the disease (*e.g.*, cancer) and characteristics of the subject, such as general health, age, sex, body weight and drug tolerance. The effective amount of the present therapeutic agent or combination to be administered will also depend on administration route and dosage form. Dosage amount and interval can be adjusted individually to provide plasma levels of the active compound that are sufficient to maintain desired therapeutic effects.

The amount of the anti-VISTA antibody or antigen-binding fragment thereof administered will depend on a variety of factors, including the nature and stage of the disease being treated (*e.g.*, cancer), the form, route and site of administration, the therapeutic regimen (*e.g.*, whether the therapeutic agent is used in combination with

immune checkpoint inhibitors), the age and condition of the particular subject being treated, the sensitivity of the patient being treated with the antibodies or the conjugates. The appropriate dosage can be readily determined by a person skilled in the art. Ultimately, a physician will determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance with normal clinical practice. The proper dosage and treatment regimen can be established by monitoring the progress of therapy using conventional techniques known to the people skilled of the art.

Effective dosages can be estimated initially from *in vitro* assays. For example, an initial dose for use in animals may be formulated to achieve a circulating blood or serum concentration of anti-VISTA antibody that is at or above the binding affinity of the antibody for VISTA as measured *in vitro*. Calculating dosages to achieve such circulating blood or serum concentrations taking into account the bioavailability of the particular antibody is well within the capabilities of skilled artisans. For guidance, the reader is referred to Fingl & Woodbury, "General Principles" in *Goodman and Gilman's The Pharmaceutical Basis of Therapeutics*, Chapter 1, latest edition, Pagamonon Press, and the references cited therein. Initial dosages can be estimated from *in vivo* data, such as animal models. Animal models useful for testing the efficacy of compounds to treat particular diseases such as cancer are generally well known in the art. Ordinarily skilled artisans can routinely adapt such information to determine dosages suitable for human administration.

The effective dose of the anti-VISTA antibody as described herein can range from about 0.001 to about 75 mg/kg per single (*e.g.*, bolus) administration, multiple administrations or continuous administration, or to achieve a serum concentration of 0.01-5000 μg/ml serum concentration per single (*e.g.*, bolus) administration, multiple administrations or continuous administration, or any effective range or value therein depending on the condition being treated, the route of administration and the age, weight and condition of the subject. In a certain embodiment, each dose can range from about 0.5 μg to about 50 μg per kilogram of body weight, for example from about 3 μg to about 30 μg per kilogram body weight.

Amount, frequency, and duration of administration will depend on a variety of factors, such as the patient's age, weight, and disease condition. A therapeutic

regimen for administration can continue for 2 weeks to indefinitely, for 2 weeks to 6 months, from 3 months to 5 years, from 6 months to 1 or 2 years, from 9 months to 18 months, or the like. Optionally, the therapeutic regimen provides for repeated administration, *e.g.*, once daily, twice daily, every two days, three days, five days, one week, two weeks, three weeks or one month. The repeated administration can be at the same dose or at a different dose. The administration can be repeated once, twice, three times, four times, five times, six times, seven times, eight times, nine times, ten times, or more. A therapeutically effective amount of anti-VISTA antibody or antigen-binding fragment thereof (optionally in combination with immune checkpoint inhibitors) can be administered as a single dose or over the course of a therapeutic regimen, *e.g.*, over the course of a week, two weeks, three weeks, one month, three months, six months, one year, or longer.

Methods of Administering the Antibody Formulations

The disclosure provides methods of prevention, treatment and/or management of a disorder, for example, a disease or disorder mediated by aberrant expression and/or activity of VISTA by administrating to a subject of an effective amount of liquid formulations of the disclosure. Various delivery systems are known and can be used to administer the liquid formulation disclosed herein. Methods of administering the present antibody liquid formulations include, but are not limited to, parenteral administration (e.g., intradermal, intramuscular, intraperitoneal, intravenous and, and subcutaneous), epidural administration, topical administration, and mucosal administration (for example, but not limited to, intranasal and oral routes). In a specific instance, the present liquid formulations are administered intramuscularly, intravenously, or subcutaneously. In one instance, the liquid formulations disclosed herein are administered subcutaneously. The formulations may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

The present disclosure also provides that a liquid formulation of the present disclosure is packaged in a hermetically sealed container such as an ampoule or sachet indicating the quantity of antibody (including antibody fragment thereof). In one embodiment, a liquid formulation of the present disclosure is in a hermetically sealed

container indicating the quantity and concentration of the antibody (including antibody fragment thereof). In one embodiment, a liquid formulation of the present disclosure is supplied in a hermetically sealed container and comprises about 1 mg/mL, about 5 mg/mL, about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 30 mg/mL, about 40 mg/mL, about 50 mg/mL, about 60 mg/mL, about 70 mg/mL,, about 80 mg/mL, about 90 mg/mL, or about 100 mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469, in a quantity of about 1 ml, about 2 ml, about 3 ml, about 4 ml, about 5 ml, 6 about ml, about 7 ml, about 8 ml, about 9 ml, about 10 ml, about 15 ml, or about 20 ml. In particular, the liquid formulation disclosed herein can be supplied in a hermetically sealed container and comprise at least about 1 mg/mL, at least about 5 mg/mL, at least about 10 mg/mL, at least about 15 mg/mL, at least about 20 mg/mL, at least about 25 mg/mL, at least about 30 mg/mL, at least about 40 mg/mL, at least about 50 mg/mL, at least about -60 mg/mL, at least about 70 mg/mL, at least about 80 mg/mL, at least about 90 mg/mL, or at least about 100 mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469, for intravenous injections, and at least about 1 mg/mL, at least about 5 mg/mL, at least about 10 mg/mL, at least about 15 mg/mL, at least about 20 mg/mL, at least about 25 mg/mL, at least about 30 mg/mL, at least about 40 mg/mL, at least about 50 mg/mL, at least about -60 mg/mL, at least about 70 mg/mL,, at least about 80 mg/mL, at least about 90 mg/mL, or at least about 100 mg/mL of an anti-VISTA antibody or a fragment thereof, for example the antibody described in WO 2022/229469, for repeated subcutaneous administration.

The amount of a liquid formulation of the present disclosure which will be effective in the prevention, treatment and/or management of a disease or disorder associated with or characterised by aberrant expression and/or activity of VISTA can be determined by standard clinical techniques well-known in the art or described herein. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the VISTA-mediated disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems, as described above.

It will be appreciated that the present anti-VISTA antibody or the antigen-binding fragment thereof can be advantageously administered with a second therapeutic agent, e.g., an immune checkpoint inhibitor as described below.

The present disclosure is further directed to a pharmaceutical composition comprising at least:

i) an anti-VISTA antibody, an antigen-binding fragment thereof, or a conjugate thereof, as disclosed herein; and

ii) a second therapeutic agent, for example an immune checkpoint inhibitor as described below,

as combination products for simultaneous, separate, or sequential use.

"Simultaneous use" as used herein refers to the administration of the two compounds of the composition according to the invention in a single and identical pharmaceutical form.

"Separate use" as used herein refers to the administration, at the same time, of the two compounds of the composition according to the invention in distinct pharmaceutical forms.

"Sequential use" as used herein refers to the successive administration of the two compounds of the composition according to the invention, each in a distinct pharmaceutical form.

Compositions of anti-VISTA antibodies (or antigen-binding fragments thereof or conjugates thereof) and second therapeutic agents, such as *e.g.*, immune checkpoint inhibitors, can be administered singly, as mixtures of one or more anti-VISTA antibodies (or antigen-binding fragments thereof or conjugates thereof) and/or one or more a second therapeutic agent (for example an immune checkpoint inhibitor as described below), in mixture or combination with other agents useful for treating cancer or adjunctive to other therapy for cancer. Examples of suitable combination and adjunctive therapies are provided below.

Encompassed by the present disclosure are pharmaceutical kits containing anti-VISTA antibodies (or antigen-binding fragments thereof or conjugates thereof) and described herein. The pharmaceutical kit is a package comprising an anti-VISTA

antibody (*e.g.*, either in lyophilised form or as an aqueous solution) and one or more of the following:

- A second therapeutic agent, for example an immune checkpoint inhibitor as described below;
- A device for administering the anti-VISTA antibody, for example a pen, needle and/or syringe; and
- Pharmaceutical grade water or buffer to resuspend the antibody if the inhibitor is in antibody form.

Each unit dose of the anti-VISTA antibody (or antigen-binding fragments thereof or conjugates thereof) can be packaged separately, and a kit can contain one or more-unit doses (*e.g.*, two-unit doses, three-unit doses, four-unit doses, five-unit doses, eight-unit doses, ten-unit doses, or more). In a specific embodiment, the one or more-unit doses are each housed in a syringe or pen.

Methods of treatment

the anti-VISTA antibody or antigen-binding fragment thereof described herein are capable of promoting T cell activation, including T cell proliferation and cytokines production, notably through activation of the effector functions of the antibody. The present formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may thus be used in methods for inducing an immune response, wherein the methods comprise administering a formulation as disclosed herein comprising an effective amount of an anti-VISTA antibody or an antigen-binding fragment thereof to a patient in need thereof. In particular, the present formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof is for use in inducing an immune response. The present disclosure also relates to the use of the present formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof for making a medicament for inducing an immune response. In a particular embodiment of the methods described herein, the induction of the immune response requires activation of the effector functions of the antibody.

The present formulations comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may be used in methods for inducing an immune response, wherein the induction of the immune response comprises inhibiting VISTA-

mediated immunosuppression, and wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. Preferably, the formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may thus be used in methods for inducing an immune response, wherein the induction of the immune response comprises promoting T cell activation, and wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. T cell activation may comprise in particular, stimulation of T cell proliferation, e.g., $CD4^+$ T cell proliferation and/or $CD8^+$ T cell proliferation, and/or cytokine production, notably proinflammatory cytokines, e.g., INF-γ, IL-2, and/or TNF-α.

The ability of the present anti-VISTA antibody to induce an immune response, *e.g.*, by promoting T cell activation, notably through induction of $CD4^+$ T cell proliferation, $CD8^+$ T cell proliferation, $CD4^+$ T cell cytokine production, and/or $CD8^+$ T cell cytokine production, thereby inhibiting VISTA-mediated immunosuppression, makes it useful for treating a variety of conditions mediated by VISTA, including cancer. Therapeutic intervention on the VISTA inhibitory pathway thus represents a promising approach to modulate inflammation and T cell-mediated immunity for the treatment of a wide variety of VISTA-mediated diseases, notably cancers. Indeed, the antibody disclosed herein inhibits tumour growth in vivo.

The formulations comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may thus be used in methods for treating VISTA-mediated diseases, notably cancer, wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. The formulation comprising an anti-VISTA antibody or antigen-binding fragment thereof described herein may thus be used in methods for treating VISTA-mediated diseases, notably cancer, wherein the treatment comprises inhibiting VISTA-mediated immunosuppression, and wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. Preferably, the formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof, described herein may thus be used in methods for treating VISTA-mediated diseases, notably cancer, wherein the treatment comprises promoting T cell

activation, and wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof.

The formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may thus be used in methods for treating VISTA-mediated diseases, notably cancer, inducing $CD4^+$ T cell proliferation, inducing $CD8^+$ T cell proliferation, inducing $CD4^+$ T cell cytokine production, and/or inducing $CD8^+$ T cell cytokine production, wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. Preferably, the formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may thus be used in methods for treating VISTA-mediated diseases, notably cancer, wherein the treatment comprises inducing $CD4^+$ T cell proliferation, inducing $CD8^+$ T cell proliferation, inducing $CD4^+$ T cell cytokine production, and/or inducing $CD8^+$ T cell cytokine production, and wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof.

Surprisingly, effector functions are required for activation of T cell by the present antibody. In contrast, a version of the humanised IgG1 anti-VISTA mAb engineered to avoid binding to human Fcγ receptors through a N298A mutation (Herbs et al. *Nature* 515(7528): 563-567), therefore devoid of any effector function, is incapable of inducing either of $CD4^+$ proliferation, $CD8^+$ proliferation, production of $CD4^+$ T cell cytokine, and production $CD8^+$ T cell cytokine. Accordingly, this variant of the anti-VISTA antibody described herein is unable to inhibit tumour proliferation in vivo.

More preferably, formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof described herein may be used in methods for treating VISTA-mediated diseases, notably cancer, wherein the treatment comprises wherein the treatment comprises promoting T cell activation by activation of the effector functions of the antibody, and wherein the methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. Even more preferably, formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof, described herein may

thus be used in methods for treating VISTA-mediated diseases, notably cancer, wherein the treatment comprises inducing $CD4^+$ T cell proliferation, inducing $CD8^+$ T cell proliferation, inducing $CD4^+$ T cell cytokine production, and/or inducing $CD8^+$ T cell cytokine production, by activation of the effector functions of the antibody, and wherein said methods comprise administering an effective amount of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof to a patient in need thereof. The therapeutic methods described herein may comprise administration of the formulations comprising the antibodies binding specifically VISTA described herein, or an antigen-binding fragments thereof, to a patient in need thereof. The VISTA antibodies and conjugates thereof, disclosed herein, are thus useful in regulating immunity, especially T cell immunity, for the treatment of VISTA-mediated diseases, notably cancer.

Accordingly, an aspect of the present disclosure relates to a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof disclosed herein for use in the treatment of a VISTA-mediated disease, notably cancer, in a patient. Also provided herein is a method of treating a VISTA-mediated disease, notably cancer, in a patient in need thereof, said method comprising the administration of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof disclosed herein to the patient. The present disclosure also relates to the use of a formulation of the anti-VISTA antibody or antigen-binding fragment thereof for making a medicament for treating a cancer.

In an embodiment, the disclosure relates to a pharmaceutical composition comprising an anti-VISTA antibody disclosed herein or an antigen-binding fragment thereof, for use in the treatment of a VISTA-mediated disease, notably cancer, in a patient. Also provided herein is a method of treating a VISTA-mediated disease, notably cancer, in a patient in need thereof, said method comprising the administration of a pharmaceutical composition comprising an anti-VISTA antibody disclosed herein or an antigen-biding fragment thereof, to the patient. The present disclosure also relates to the use of a pharmaceutical composition comprising an anti-VISTA antibody disclosed herein or an antigen-biding fragment, for making a medicament for treating a VISTA-mediated disease, notably cancer.

Cancer that can be treated with the formulations or pharmaceutical compositions comprising the antibody disclosed herein can include any malignant or

benign tumour of any organ or body system. Examples include, but are not limited to, the following: breast, digestive/gastrointestinal, endocrine, neuroendocrine, eye, genitourinary, germ cell, gynaecologic, head and neck, hematologic/blood, musculoskeletal, neurologic, respiratory/thoracic, bladder, colon, rectal, lung, endometrial, kidney, pancreatic, salivary gland, liver, stomach, peritoneal, testicular, oesophageal, prostate, brain, cervical, ovarian and thyroid cancers. Other cancers can include melanomas, mesothelioma, sarcomas, glioblastoma, haematological cancers such as leukaemia, myelomas, and lymphomas, and any cancer described herein. In some embodiments, the solid tumour is infiltrated with myeloid and/or T-cells. In some embodiments, the cancer is a leukaemia, lymphoma, myelodysplastic syndrome, mesothelioma, and/or myeloma. In some embodiments, the cancer can be any kind or type of leukaemia, including a lymphocytic leukaemia or a myelogenous leukaemia, such as, e.g., acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), acute myeloid (myelogenous) leukaemia (AML), chronic myelogenous leukaemia (CML), hairy cell leukaemia, T-cell prolymphocytic leukaemia, large granular lymphocytic leukaemia, or adult T-cell leukaemia. In some embodiments, the lymphoma is a histocytic lymphoma, follicular lymphoma or Hodgkin lymphoma, and in some embodiments, the cancer is a multiple myeloma. In some embodiments, the cancer is a solid tumour, for example, a melanoma, or bladder cancer. In a particular embodiment, the cancer is a lung cancer, such as a non-small cell lung cancer (NSCLC). The present invention also provides a method for modulating or treating at least one malignant disease in a cell, tissue, organ, animal or patient, including, but not limited to, at least one of: leukaemia, acute leukaemia, acute lymphoblastic leukaemia (ALL), B-cell, T-cell or FAB ALL, acute myeloid leukaemia (AML), chronic myelocytic leukaemia (CML), chronic lymphocytic leukaemia (CLL), hairy cell leukaemia, myelodysplastic syndrome (MDS), a lymphoma, Hodgkin's disease, a malignant lymphoma, non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, Kaposi's sarcoma, colorectal carcinoma, pancreatic carcinoma, nasopharyngeal carcinoma, malignant histiocytosis, paraneoplastic syndrome/hypercalcemia of malignancy, solid tumours, adenocarcinomas, sarcomas, malignant melanoma, haemangioma, metastatic disease, cancer related bone resorption, cancer-related bone pain, and the like. In some embodiments, the solid tumour is infiltrated with myeloid and/or T-cells. In a particular embodiment, the solid tumour is a lung cancer, such as a non-small cell lung cancer (NSCLC). In another embodiment, the solid tumour is mesothelioma.

Preferably, the cancer is selected in the group consisting of the cancer bladder cancer, breast cancer, cervical cancer, colon cancer, endometrial cancer, oesophageal cancer, fallopian tube cancer, gall bladder cancer, gastrointestinal cancer, head-and-neck cancer, haematological cancer (e.g., leukaemia, lymphoma, or myeloma), laryngeal cancer, liver cancer, lung cancer, lymphoma, melanoma, mesothelioma, ovarian cancer, primary peritoneal cancer, salivary gland cancer, sarcoma, stomach cancer, thyroid cancer, pancreatic cancer, renal cell carcinoma, glioblastoma, and prostate cancer.

The present antibody is particularly useful because it can induce an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient, as detailed above. Thus, in an embodiment, formulations comprising the anti-VISTA antibody or antigen-binding fragment thereof is for use in the treatment of a VISTA-mediated disease, notably cancer, in a patient, wherein the use comprises inducing an immune response in the patient. Also provided herein is a method of treating a VISTA-mediated disease, notably cancer, in a patient in need thereof, the method comprising administering the formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof disclosed herein to the patient and inducing an immune response in this patient. The present disclosure also relates to the use of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof for making a medicament for treating a VISTA-mediated diseases, notably cancer, wherein the treatment comprises inducing an immune response in the patient.

In an embodiment, the disclosure relates to a pharmaceutical composition as disclosed herein, wherein the pharmaceutical composition comprises the present anti-VISTA antibody or antigen-binding fragment thereof, for use in the treatment of a VISTA-mediated diseases, notably cancer, in a patient, wherein the use comprises inducing an immune response in the patient. Also provided herein is a method of treating VISTA-mediated diseases, notably cancer, in a patient in need thereof, the method comprising administering a pharmaceutical composition comprising the anti-VISTA antibody or antigen-binding fragment thereof disclosed herein to the patient and inducing an immune response in this patient. The present disclosure also relates to the use of a pharmaceutical composition disclosed herein, wherein the pharmaceutical composition comprises the present anti-VISTA antibody or antigen-binding fragment thereof, for making a medicament for treating a VISTA-mediated disease, notably cancer, wherein the treatment comprises inducing an immune response in the patient.

An embodiment provides a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof for use in inducing an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient. Also provided herein is a method of inducing an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient, in need thereof, said method comprising the administration of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof disclosed herein to the patient. The present disclosure also relates to the use of a formulation comprising the anti-VISTA antibody or antigen-binding fragment thereof for making a medicament for inducing an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient.

In an embodiment, the disclosure relates to a pharmaceutical composition comprising an anti-VISTA antibody disclosed herein or a conjugate thereof, for use in inducing an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient. Also provided herein is a method of an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient, in need thereof, said method comprising the administration of a pharmaceutical composition comprising an anti-VISTA antibody disclosed herein or a conjugate thereof, to the patient. The present disclosure also relates to the use of a pharmaceutical composition comprising an anti-VISTA antibody disclosed herein or a conjugate thereof, for making a medicament for inducing an immune response in a patient having a VISTA-mediated disease, *e.g.*, a cancer patient.

The immune response thus generated by the antibody disclosed herein includes, without limitation, induction of $CD4^+$ T cell proliferation, induction of $CD8^+$ T cell proliferation, induction of $CD4^+$ T cell cytokine production, and induction of $CD8^+$ T cell cytokine production. Preferably, the effector functions are required for the antibody disclosed herein to generate the immune response, including, without limitation, induction of $CD4^+$ T cell proliferation, induction of $CD8^+$ T cell proliferation, induction of $CD4^+$ T cell cytokine production, and induction of $CD8^+$ T cell cytokine production.

The anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, may be admixed with a second therapeutic agent in the present formulations.

A "therapeutic agent" encompasses biological agents, such as an antibody, a peptide, a protein, an enzyme, and chemotherapeutic agents. The therapeutic agent

also encompasses immuno-conjugates of cell-binding agents (CBAs) and chemical compounds, such as antibody-drug conjugates (ADCs). The drug in the conjugates can be a cytotoxic agent, such as one described herein.

As used herein, the anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, and the other therapeutic agent are said to be administered successively if they are administered to the patient on the same day, for example during the same patient visit. Successive administration can occur 1, 2, 3, 4, 5, 6, 7 or 8 hours apart. In contrast, the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, of the disclosure and the other therapeutic agent are said to be administered separately if they are administered to the patient on the different days, for example, the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, of the disclosure and the other therapeutic agent can be administered at a 1-day, 2-day or 3-day, one-week, 2-week or monthly intervals. In the methods of the present disclosure, administration of the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, of the disclosure can precede or follow administration of the other therapeutic agent.

As a non-limiting example, the anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, and other therapeutic agent can be administered concurrently for a period of time, followed by a second period of time in which the administration of the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, of the disclosure and the other therapeutic agent is alternated.

Combination therapies of the present disclosure can result in a greater than additive, or a synergistic, effect, providing therapeutic benefits where neither the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, nor the other therapeutic agent is administered in an amount that is, alone, therapeutically effective. Thus, such agents can be administered in lower amounts, reducing the possibility and/or severity of adverse effects.

In a preferred embodiment, the other therapeutic agent is a chemotherapeutic agent. Said chemotherapeutic agent is preferably an alkylating agent, an anti-metabolite, an anti-tumour antibiotic, a mitotic inhibitor, a chromatin function inhibitor, an anti-angiogenesis agent, an anti-oestrogen, an anti-androgen or an immunomodulator.

The term "alkylating agent," as used herein, refers to any substance which can cross-link or alkylate any molecule, preferably nucleic acid (*e.g.*, DNA), within a cell. Examples of alkylating agents include nitrogen mustard such as mechlorethamine, chlorambucol, melphalen, chlorydrate, pipobromen, prednimustin, disodic-phosphate or estramustine; oxazophorins such as cyclophosphamide, altretamine, trofosfamide, sulfofosfamide or ifosfamide; aziridines or imine-ethylènes such as thiotepa, triethylenamine or altetramine; nitrosourea such as carmustine, streptozocin, fotemustin or lomustine; alkyle-sulfonates such as busulfan, treosulfan or improsulfan; triazenes such as dacarbazine; or platinum complexes such as cis-platinum, oxaliplatin and carboplatin.

The expression "anti-metabolites," as used herein, refers to substances that block cell growth and/or metabolism by interfering with certain activities, usually DNA synthesis. Examples of anti-metabolites include methotrexate, 5-fluoruracil, floxuridine, 5-fluorodeoxyuridine, capecitabine, cytarabine, fludarabine, cytosine arabinoside, 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), chlorodesoxyadenosine, 5-azacytidine, gemcitabine, cladribine, deoxycoformycin and pentostatin.

As used herein, "anti-tumour antibiotics" are compounds which may prevent or inhibit DNA, RNA and/or protein synthesis. Examples of anti-tumour antibiotics include doxorubicin, daunorubicin, idarubicin, valrubicin, mitoxantrone, dactinomycin, mithramycin, plicamycin, mitomycin C, bleomycin, and procarbazine.

"Mitotic inhibitors," as used herein, prevent normal progression of the cell cycle and mitosis. In general, microtubule inhibitors or taxoids such as paclitaxel and docetaxel are capable of inhibiting mitosis. Vinca alkaloid such as vinblastine, vincristine, vindesine and vinorelbine are also capable of inhibiting mitosis.

As used herein, the terms "chromatin function inhibitors" or "topoisomerase inhibitors" refer to substances which inhibit the normal function of chromatin modelling proteins such as topoisomerase I or topoisomerase II. Examples of chromatin function inhibitors include, for topoisomerase I, camptothecine and its derivatives such as topotecan or irinotecan, and, for topoisomerase II, etoposide, etoposide phosphate and teniposide.

As used herein, the term "anti-angiogenesis agent" refers to any drug, compound, substance or agent which inhibits growth of blood vessels. Exemplary anti-

angiogenesis agents include, but are by no means limited to, razoxin, marimastat, batimastat, prinomastat, tanomastat, ilomastat, CGS-27023A, halofuginon, COL-3, neovastat, BMS-275291, thalidomide, CDC 501, DMXAA, L-651582, squalamine, endostatin, SU5416, SU6668, interferon-alpha, EMD121974, interleukin-12, IM862, angiostatin and vitaxin.

As used herein, the terms "anti-oestrogen" or "anti-estrogenic agent" refer to any substance which reduces, antagonizes or inhibits the action of oestrogen. Examples of anti-oestrogen agents are tamoxifen, toremifene, raloxifene, droloxifene, iodoxyfene, anastrozole, letrozole, and exemestane.

As used herein, the terms "anti-androgens" or "anti-androgen agents" refer to any substance which reduces, antagonises or inhibits the action of an androgen. Examples of anti-androgens are flutamide, nilutamide, bicalutamide, sprironolactone, cyproterone acetate, finasteride and cimitidine.

"Immunomodulators" as used herein are substances which stimulate the immune system.

Examples of immunomodulators include interferon, interleukin such as aldesleukine, OCT-43, denileukin diflitox and interleukin-2, tumoural necrose fators such as tasonermine or others immunomodulators such as lentinan, sizofiran, roquinimex, pidotimod, pegademase, thymopentine, poly I:C or levamisole in conjunction with 5-fluorouracil.

For more detail, the person of skill in the art can refer to the manual edited by the "Association Française des Enseignants de Chimie Thérapeutique" and entitled "Traité de chimie thérapeutique", vol. 6, Médicaments antitumouraux et perspectives dans le traitement des cancers, edition TEC & DOC, 2003.

It can also be mentioned as chemical agents or cytotoxic agents, all kinase inhibitors such as, for example, gefitinib or erlotinib.

More generally, examples of suitable chemotherapeutic agents include but are not limited to 1-dehydrotestosterone, 5-fluorouracil decarbazine, 6-mercaptopurine, 6-thioguanine, actinomycin D, adriamycin, aldesleukin, alkylating agents, allopurinol sodium, altretamine, amifostine, anastrozole, anthramycin (AMC)), anti-mitotic agents, cis-dichlorodiamine platinum (II) (DDP) cisplatin), diamino dichloro platinum,

anthracyclines, antibiotics, antimetabolites, asparaginase, BCG live (intravesical), betamethasone sodium phosphate and betamethasone acetate, bicalutamide, bleomycin sulfate, busulfan, calcium leucouorin, calicheamicin, capecitabine, carboplatin, lomustine (CCNU), carmustine (BSNU), Chlorambucil, Cisplatin, Cladribine, Colchicin, conjugated estrogens, Cyclophosphamide, Cyclothosphamide, Cytarabine, Cytarabine, cytochalasin B, Cytoxan, Dacarbazine, Dactinomycin, dactinomycin (formerly actinomycin), daunirubicin HCL, daunorucbicin citrate, denileukin diftitox, Dexrazoxane, Dibromomannitol, dihydroxy anthracin dione, Docetaxel, dolasetron mesylate, doxorubicin HCL, dronabinol, *E. coli* L-asparaginase, emetine, epoetin-α, Erwinia L-asparaginase, esterified estrogens, estradiol, estramustine phosphate sodium, ethidium bromide, ethinyl estradiol, etidronate, etoposide citrororum factor, etoposide phosphate, filgrastim, floxuridine, fluconazole, fludarabine phosphate, fluorouracil, flutamide, folinic acid, gemcitabine HCL, glucocorticoids, goserelin acetate, gramicidin D, granisetron HCL, hydroxyurea, idarubicin HCL, ifosfamide, interferon α-2b, irinotecan HCL, letrozole, leucovorin calcium, leuprolide acetate, levamisole HCL, lidocaine, lomustine, maytansinoid, mechlorethamine HCL, medroxyprogesterone acetate, megestrol acetate, melphalan HCL, mercaptipurine, mesna, methotrexate, methyltestosterone, mithramycin, mitomycin C, mitotane, mitoxantrone, nilutamide, octreotide acetate, ondansetron HCL, oxaliplatin, paclitaxel, pamidronate disodium, pentostatin, pilocarpine HCL, plimycin, polifeprosan 20 with carmustine implant, porfimer sodium, procaine, procarbazine HCL, propranolol, rituximab, sargramostim, streptozotocin, tamoxifen, taxol, tegafur, teniposide, tenoposide, testolactone, tetracaine, thioepa chlorambucil, thioguanine, thiotepa, topotecan HCL, toremifene citrate, trastuzumab, tretinoin, valrubicin, vinblastine sulfate, vincristine sulfate, and vinorelbine tartrate.

The anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, as disclosed herein can be administered to a patient in need of treatment for cancer receiving a combination of chemotherapeutic agents. Exemplary combinations of chemotherapeutic agents include 5-fluorouracil (5FU) in combination with leucovorin (folinic acid or LV); capecitabine, in combination with uracil (UFT) and leucovorin; tegafur in combination with uracil (UFT) and leucovorin; oxaliplatin in combination with 5FU, or in combination with capecitabine; irinotecan in combination with capecitabine, mitomycin C in combination with 5FU, irinotecan or capecitabine. Use of other combinations of chemotherapeutic agents disclosed herein is also possible.

The anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, can also be combined with other therapeutic antibodies. Accordingly, therapy based on the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof, disclosed herein can be combined with, or administered adjunctive to a different monoclonal antibody such as, for example, but not by way of limitation, an anti-EGFR (EGF receptor) monoclonal antibody or an anti-VEGF monoclonal antibody. Specific examples of anti-EGFR antibodies include cetuximab and panitumumab. A specific example of an anti-VEGF antibody is bevacizumab.

Notably, the therapeutic methods described herein may comprise the administration of an immune checkpoint inhibitor along with the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof. The immune checkpoint inhibitor and the anti-VISTA antibody, or antigen-binding fragment or conjugate thereof may be administered simultaneously, separately, or sequentially.

As used herein, a "checkpoint inhibitor" refers to a molecule, such as *e.g.*, a small molecule, a soluble receptor, or an antibody, which targets an immune checkpoint and blocks the function of said immune checkpoint. More specifically, a "checkpoint inhibitor" as used herein is a molecule, such as *e.g.*, a small molecule, a soluble receptor, or an antibody, that blocks certain proteins made by some types of immune system cells, such as T cells, and some cancer cells.

In a first embodiment, the immune checkpoint inhibitor is an inhibitor of any one of CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIGIT, TIM3, GAL9, LAG3, PSG-L1, VSIG4, KIR, 2B4 (belongs to the CD2 family of molecules and is expressed on all NK, γδ, and memory CD8+ (αß) T cells), CD160 (also referred to as BY55), CGEN-15049, CHK 1 and CHK2 kinases, IDO1, A2aR and any of the various B-7 family ligands.

Exemplary immune checkpoint inhibitors include anti-CTLA-4 antibody (*e.g.*, ipilimumab), anti-LAG-3 antibody (*e.g.*, BMS-986016), anti-B7-H3 antibody, anti-B7-H4 antibody, anti-Tim3 antibody (*e.g.*, TSR-022, MBG453), anti-BTLA antibody, anti-KIR antibody, anti-A2aR antibody, anti CD200 antibody, anti-PD-1 antibody (*e.g.*, pembrolizumab, nivolumab, cemiplimab, pidilizumab), anti-PD-L1 antibody (*e.g.*, atezolizumab, avelumab, durvalumab, BMS 936559), anti-TIGIT antibody (*e.g.*, tiragolumab, vibostolimab), anti-VSIG4 antibody, anti-CD28 antibody, anti-CD80 or -CD86 antibody, anti-B7RP1 antibody, anti-B7-H3 antibody, anti-HVEM antibody, anti-CD137 antibody (*e.g.*, urelumab), anti-CD137L antibody, anti-OX40 (*e.g.*, 9B12, PF-

04518600, MEDI6469), anti-OX40L antibody, anti-CD40 or -CD40L antibody, anti-GAL9 antibody, anti-IL-10 antibody, fusion protein of the extracellular domain of a PD-1 ligand, *e.g.* PDL-1 or PD-L2, and IgG1 (*e.g.*, AMP-224), fusion protein of the extracellular domain of a OX40 ligand, *e.g.* OX40L, and IgG1 (*e.g.*, MEDI6383), IDO1 drug (*e.g.*, epacadostat) and A2aR drug. A number of immune checkpoint inhibitors have been approved or are currently in clinical trials. Such inhibitors include ipilimumab, pembrolizumab, nivolumab, cemiplimab, pidilizumab, atezolizumab, avelumab, durvalumab, tiragolumab, vibostolimab, BMS 936559, urelumab, 9B12, PF-04518600, BMS-986016, TSR-022, MBG453, MEDI6469, MEDI6383, and epacadostat.

Examples of immune checkpoints inhibitors are listed for example in Marin-Acevedo et al., *Journal of Haematology & Oncology* **11**: 8, 2018; Kavecansky and Pavlick, *AJHO* **13**(2): 9-20, 2017; Wei et al., *Cancer Discov* **8**(9): 1069-86, 2018.

Preferably, the immune checkpoint inhibitor is an inhibitor of CTLA-4, LAG-3, Tim3, PD-1, PD-L1, PSG-L1, VSIG4, CD137, OX40, or IDO1. More preferably, the immune checkpoint inhibitor is an inhibitor of PD-1 or PD-L1. Even more preferably, the immune checkpoint inhibitor is an antibody inhibiting PD-1 or an antibody inhibiting PD-L1.

Accordingly, the present disclosure preferably relates to a combination therapy of an anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, and an anti-PD-1 antibody or an anti-PD-L1 antibody for treating a VISTA-mediated disease, notably cancer. In a first aspect, the present anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, is for use in treating a VISTA-mediated disease, notably cancer, wherein the treatment comprises further administrating an anti-PD-1 or an anti-PD-L1 antibody. The present disclosure also relates to a method of treating a VISTA-mediated disease, notably cancer, comprising administering an effective amount of the present anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, and an effective amount of an anti-PD-1 or anti-PD-L1 antibody to a subject in need thereof. In another aspect, the present disclosure also relates to the use of the anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof disclosed herein for making a medicament for treating a VISTA-mediated disease, notably cancer, wherein the treatment comprises administering an anti-PD-1 antibody or an anti-PD-L1 antibody.

The anti-VISTA antibody or antigen-binding fragment thereof or conjugate thereof, and the anti-PD-1 or anti-PD-L1 antibody may be administered simultaneously, separately, or sequentially.

Hereinbelow, the present invention is explained in detail in view of the examples. However, the following examples are given only for exemplification of the present invention, and it is evident that the present invention is not limited to the following examples.

EXAMPLES

Example 1: Identification of deamidation sites in Ab3

The monoclonal antibody Ab3 was originally disclosed in WO2016/94837. Ab3 comprises a heavy chain of sequence SEQ ID NO:11 and a light chain of sequence SEQ ID NO:12. Bioinformatic analysis predicts that there are 2 potential deamidation sites in the light chain and 9 in the heavy chain of Ab3.

In order to investigate whether any of these sites actually undergo deamidation, Ab3 was subjected to Cationic Exchange Chromatography (CEX) as described in Goyon et al. (*J Chromatogr B Analyt Technol Biomed Life Sci.* **1065-1066**:119-128 (2017)).

Method: *CEX (pH gradient)*

Material:

- Column MabPac SCX-10 4x250mm, 10µm (Thermo,ref : 74625)
- Eluent:
    - Buffer A: CX-1 pH gradient buffer A pH 5.6 (Thermo, ref: 85349)
    - Buffer B: CX-1 pH gradient buffer B pH 10.2 (Thermo, ref: 85349)
    - Buffers are diluted at 1/10 with MilliQ water and filtered / 0.22µm (for extemporaneous use)
    - Sample preparation & method:
- Sample are diluted at 1mg/mL with MilliQ water
- Injection of 20 µL diluted sample

- Gradient: see Table 3.

Table 3: Gradient used

Time (min)	Flow (mL/min)	% Eluent A	% Eluent B
0	1	100	0
1	1	100	0
31	1	0	100
34	1	0	100
35	1	100	0
45	1	100	0

Results

As shown on Fig. 1, Ab3 is a highly heterogenous mixture of 3 main charges variants with 40 % of heavy chain N55/N55, 33% of N55 and D55 de-amidated variant, and 8% of full D55 deamidated variant. This result was confirmed by structure assessment (LC-MS). Forced degradation studies (pH9, 40°C, 3 days; vs pembrolizumab) led to the same conclusion: when the Asn residues of the VL and VH chains of Ab3 are examined under these conditions, degradation is only observed for N55 in the heavy chain while the other Asn residues do not appear to be affected. Therefore, in all these different experimental conditions, position N55 in the heavy chain was identified as the major, if not sole site of deamidation in Ab3.

Example 2: Generation and characterisation of Ab1

Based on the results of example 1, a variant of Ab3 was created by mutating the Asn at position 55 in the heavy chain into an Asp. This variant was designated Ab1.

Ab1 is an anti-VISTA humanised monoclonal antibody based on a human Immunoglobulin G1 (IgG1 k; G1m3 (R215) allotype) framework. The recombinant

antibody is produced in Chinese Hamster Ovary (CHO) cells and consists of two heavy chains (HC) of 448 amino acid residues each and two kappa light chains (LC) of 213 amino acid residues each with typical IgG1 inter and intra chain disulfide bonds.

The structure, physicochemical characteristics, immunological and biological properties of anti-VISTA antibody Ab1 were established using a comprehensive set of methods:

- Molecular weight: 147213 Da (G0F/G0F, pE/pE, 16 disulfide bridges)
- Molecular formula: $C_{6410}H_{9904}N_{1686}O_{2009}S_{50}$
- N-glycosylation sites: 298, 298"
- Disulfide bridges are located:
  - Intra-chain (light chain): Cys(23) to Cys(87); Cys(133) to Cys(193)
  - Intra-chain (heavy chain): Cys(22) to Cys(96); Cys(145) to Cys(201); Cys(262) to Cys(322); Cys(368) to Cys (426)
  - Inter-chain (light chain and heavy chain): Cys(213)LC to Cys(221)HC; Cys(227)HC to Cys(227)HC; Cys(230)HC to Cys(230)HC

The expected average molar mass of the full-length IgG, the deglycosylated IgG, the IdeS digested and reduced IgGs were confirmed. The N-terminal residue of the heavy chain is encoded as a glutamine but exists mainly in the pyroglutamic acid form. There is one N-glycosylation site on the heavy chain (Asn298), and it is predominantly occupied with a typical core fucosylated biantennary glycan with 0, 1 or 2 terminal galactose residues as expected for CHO produced recombinant IgGs. Most of the C-terminal lysine residues in the heavy chains are clipped.

The molecular weights are presented in Table 4 below:

Table 4: Determination of Ab1 molecular weights

Whole antibody mass (LC-UV-MS) (Da) Expected mass: 147 213 Da

Deglycosylated antibody mass (LC-UV-MS) (Da) Expected mass: 145 020 Da
Subunits profile, middle level (LC-UV-MS) (IdeS/reduced) (Da) Light chain: Expected mass: 23 380 Da Fd fragment: Expected mass: 25 024 Da Fc/2 fragment Expected mass: 25 236 Da

Example 3: Determination of Ab1 binding to VISTA

Mutations in the CDR are known to affect the binding efficacy of antibodies. For example, a 400-fold decrease in antigen binding affinity was observed when Asn33 in the CRDL1 of an anti-CD52 a monoclonal antibody was replaced with an Asp (Qiu et al. *mAbs*. **11**(7): 1266-1275 (2019)).

Ab1 binding to recombinant human (rh) VISTA-His protein VISTA was investigated by direct and indirect ELISA. In direct ELISA, the rhVISTA-His protein was directly immobilised on the plate, whilst in indirect ELISA, the rhVISTA-His protein was captured using an immobilised anti-His antibody.

Antibodies tested are provided in Table 5.

Table 5: Antibodies tested in ELISA

Antibody tested	Reference	Antibody concentration determined by HPLC (mg/ml)
Ab3 - Batch 1 (unmutated antibody)	1109171	10.5
Ab3 - Batch 2 (unmutated antibody)	MID1-21	10.6
Ab1 (N55D substitution in the CDR-H2)	MID1-86B	1.63

IgG1 anti-VISTA antibody with deletion of lysine at C-terminal (positive control)	MID1-34A	1.87
Anti-hVISTA rabbit polyclonal antibody (positive control)	SinoBio #13482-T16-100	1
c9G4 (IgG1) / (negative control)	F58000.018.13094wfb	7,45

The anti-VISTA antibody Ab1 with an Asp at position 55 was compared with two different batches of Ab3 antibody (which has an Asn at position 55) as well as to IgG1 anti-VISTA and anti-hVISTA rabbit polyclonal antibody (positive controls) and an irrelevant c9G4 antibody (negative control).

Direct ELISA

Methods

Wells were coated overnight at 4°C with 100 µl of rhVISTA at 0.3 µg/ml in 1x D-PBS.

After incubation, the coating solution was removed and plates were blocked by adding 250 µl blocking buffer (0.5% gelatin in 1x PBS) for at least 1 hour at 37°C.

After blocking, primary antibody (among those listed in Table 3) in dilution buffer (1x PBS + 0.1% gelatin + 0.05% Tween 20) was serially diluted 1:3 from an initial concentration of 5 µg/mL such that each well had a final volume of 100 µL and was incubated for 1 hour at 37°C.

Wells were washed 3x with 300 µl 1x PBS

100 µl secondary antibody (AffiniPure goat anti-rabbit specific IgG (H+L) HRP (Immuno Research Jackson ref. 111-035-003) or AffiniPure goat anti-human specific IgG (Fc fragment) HRP (Immuno Research Jackson #109-035-098), diluted 1:5000 in the dilution buffer was added to wells, and was incubated for 1 hour at 37°C.

Wells were washed 3x with 300 µl 1x PBS

100µL TMB was added to each well and plates were incubated for 5 min at room temperature. Reaction was stopped with addition of 100 µl of 1 M H_2SO_4 per well and absorbance was read at 450 nm with a microplate reader.

Results

While it has been reported that several IgG1 monoclonal antibodies lose activity as a result of deamidation, binding affinity of the Ab1 anti-VISTA antibody was unexpectedly maintained. Indeed, Ab1 had a very similar profile when compared to the unmutated antibody Ab3 (see Fig. 2). EC_{50} values were approximately $8.83x10^{-10}$ M (CV 21%) (Table 6), in contrast to what had previously been observed. As expected, the c9G4 negative control antibody showed no binding.

Table 6: EC_{50} values obtained for the tested antibodies in each of three experiments (n1, n2, and n3) in rhVISTA direct ELISA.

	Direct ELISA	EC_{50}			MEAN EC_{50} (M)	STANDARD DEVIATION (M)	CV (%)	EC_{50} "Prism" n=3	Mean CV "Prism" (%)
	Antibody	n1	n2	n3					
1	Ab1	1,04E-09	1,08E-09	9,28E-10	1,02E-09	7,92E-11	7,8	1,02E-09	5,9
2	Ab3 batch 1	1,10E-09	8,77E-10	9,01E-10	9,61E-10	1,25E-10	13,0	9,59E-10	6,9
3	Ab3 batch 2	6,41E-10	7,28E-10	6,45E-10	6,71E-10	4,93E-11	7,3	6,71E-10	5,2
4	IgG1 anti-VISTA	4,74E-10	4,79E-10	4,18E-10	4,57E-10	3,40E-11	*7,4*	4,58E-10	5,4
5	Anti-hVISTA rabbit polyclonal antibody	5,50E-11	5,17E-11	6,13E-11	5,60E-11	4,87E-12	8,7	5,59E-11	4,3
				MEAN (1/2/3)	8,83E-10	1,86E-10	21		

Indirect ELISA

Methods

Wells were coated overnight at 4°C with 100 µl of an anti-6x Histidine mouse monoclonal IgG1, clone AD1.1.10 (RD systems cat#MAB050) at 2 µg/ml in 1x D-PBS (indirect ELISA).

After incubation, the coating solution was removed and plates were blocked by adding 250 µl blocking buffer (0.5% gelatine in 1x PBS) for at least 1 hour at 37°C.

After blocking, 100 µL of rhVISTA at 0.3 µg/ml in dilution buffer (1x PBS + 0.1% gelatine + 0.05% Tween 20) was added to each well and incubated for 1 hour at 37°C.

Wells were washed 3x with 300 µl 1x PBS

Primary antibody (among those listed in Table 3) in dilution buffer was serially diluted from an initial concentration of 5 µg/mL such that each well had a final volume of 100 µL and was incubated for 1 hour at 37°C.

Wells were washed 3x with 300 µl 1x PBS

100µl secondary antibody (AffiniPure goat anti-rabbit specific IgG (H+L) HRP (Immuno Research Jackson ref. 111-035-003) or AffiniPure goat anti-human specific IgG (Fc fragment) HRP (Immuno Research Jackson #109-035-098), diluted 1:5000 in the dilution buffer was added to wells, and was incubated for 1 hour at 37°C.

Wells were washed 3x with 300 µl 1x PBS

100µL TMB was added to each well and plates were incubated for 5 min at room temperature. Reaction was stopped with addition of 100 µl of 1 M H_2SO_4 per well and absorbance was read at 450 nm with a microplate reader.

Results

While it has been reported that several IgG1 monoclonal antibodies have reduced affinity as a result of deamidation, the affinity of the Ab1 anti-VISTA antibody was unexpectedly maintained here. Indeed, Ab1 had a very similar profile when compared to the unmutated antibody Ab3 (see Fig. 3). EC_{50} values were approximately $4.20x10^{-11}$ M (CV 10%) (Table 7). As expected, the c9G4 negative control antibody showed no binding.

Table 7: EC_{50} values obtained for the tested antibodies in each of three experiments (n1, n2, and n3) in rhVISTA indirect ELISA

Indirect ELISA	EC_{50}			CV (%)		

	Antibody	n1	n2	n3	MEAN EC_{50} (M)	STANDARD DEVIATION (M)		EC_{50} "Prism" n=3	Mean CV "Prism" (%)
1	Ab1	5,01E-11	4,47E-11	4,65E-11	4,71E-11	2,75E-12	5,8	4,71E-11	6,6
2	Ab3 batch 1	4,58E-11	3,42E-11	3,86E-11	3,96E-11	5,85E-12	14,8	3,93E-11	6,0
3	Ab3 batch 2	3,82E-11	4,18E-11	3,80E-11	3,93E-11	2,12E-12	5,4	3,93E-11	3,7
4	IgG1 anti-VISTA	2,63E-11	2,59E-11	3,29E-11	2,83E-11	3,93E-12	13,9	2,82E-11	5,2
5	Anti-hVISTA rabbit polyclonal antibody	4,07E-10	3,50E-10	6,70E-10	4,76E-10	1,71E-10	35,9	4,53E-10	16,4
				MEAN (1/2/3)	4,20E-11	4,39E-12	10		

Example 4: Evaluation of T cells activation and cytokines release in CHO-VISTA coculture with PBMC

VISTA is known to be an immune checkpoint protein that critically regulates immune responses. Since Ab1 binds VISTA with the same affinity as the original antibody Ab3, it was investigated whether Ab1 was capable, like Ab3, to reverse that immune suppression.

A schematic representation of the experiment is shown in Fig. 4.

Chinese Hamster Ovary (CHO) cells WT or transfected to express human VISTA protein were irradiated with Faxitron X-ray machine 90Gy to reduce their proliferation and metabolism.

20,000 CHO cells were then cultured with 200,000 PBMC cells in 96 well plates (ratio CHO:PBMC = 1:10).

The mixture was then incubated at 37°C and 5% CO_2 with anti-CD3/CD28 beads (ratio: 1 bead for 32 cells) in presence of Ab1 or a hIgG1 negative control 10µg/mL in a total of 200µl/96 wells.

At day 3, supernatants were recovered and analysed by MSD (Meso Scale Discovery) for cytokines release and by flow cytometry (FACS) for the CD25 T cells activation marker expression on CD4 and CD8 T cells.

As expected, proliferation of $CD4^+$ and $CD8^+$ T cells was suppressed in the presence of CHO-VISTA. This suppression was reversed by the addition of the antibody Ab1. In the presence of Ab1, strong proliferation of both $CD4^+$ and $CD8^+$ T cells could be observed. However, no such stimulation was detected with the negative control hIgG1 antibody. Likewise, addition of Ab1 to the mixture of PBMCs and CHO-VISTA triggered a strong production of IFNγ, IL-2, and TNFα, confirming the activation of the $CD4^+$ and $CD8^+$ T cells (Fig. 5). Once again, the hIgG1 negative control showed no effect. These results thus demonstrate that Ab1 has retained an activity inhibiting VISTA-mediated immunosuppression.

In an attempt to understand the mechanism of this inhibition, a mutation was introduced at position 298 (N298A) in the Fc domain of Ab1. Antibodies with this mutation are known to be unable to activate effector functions, *e.g.*, ADCC, CDC, and ADCP, as this mutation eliminates their ability to bind to human Fcγ receptors (see *e.g.* Liu et al. *Antibodies (Basel)*. **9**(4): 64.(2020); Herbst et al. *Nature*. **515**(7528):563-567 (2014)).

Surprisingly, the mutation completely abolished Ab1 ability to induce $CD4^+$ and $CD8^+$ T cell proliferation. Likewise, no cytokine release could be detected when the Asn298 was replaced with an alanine. These results thus indicate that the interaction of Ab1 with the Fcγ receptors -and thus the effector functions of Ab1 - are crucial for the Ab1 reversal of VISTA immunosuppression (Fig. 5).

The negative control was not affected by the introduction of the same mutation in its Fc.

VISTA blockade by Ab1 thus reverses immune suppression. This activity requires the effector functions (ADCC and/or CDC and/or ADCP) of the antibody.

Example 5: Ab1 inhibits VISTA binding to PSG-L1 and VSIG3.

Several VISTA ligands have been described. In particular, VSIG3 has been identified as a major ligand for VISTA demonstrating specific binding and functional in vitro inhibition of T cell activation (Wang et al. *Immunology*. **156**(1):74-85 (2019)). In

addition, the pH-dependent binding of VISTA to P-selectin glycoprotein ligand-1 (PSGL-1) has been described; blockade of this interaction in acidic environment is sufficient to reverse VISTA mediated immune suppression in vivo (Johnston et al. *Nature.* **574**(7779): 565-570. (2019)).

It was therefore investigated whether Ab1 could block the interaction between VISTA and VSIG3 and/or the interaction of VISTA with PSG-L1 in an acidic pH environment (pH = 6).

Evaluation of rhVISTA-His (monomer) or rhVISTA-Fc (dimer) binding on rhVSIG3-Fc grafted on a CM5 sensor chip (2200 RU).

The interaction was measured by Biacore at pH=7.4

rhVISTA-His (monomer) and rhVISTA-Fc (dimer) were tested at 700nM in presence of a range of concentrations (0 to 1200 nM) of the anti-VISTA Ab1.

Fig. 6 shows the dose response curve of VISTA-VSIG3 binding in presence of Ab1 compared to VISTA-VSIG3 binding in absence of Ab1 (100 % binding). Ab1 disrupts the VISTA-VSIG3 interaction in dose dependent manner.

Evaluation of anti-VISTA antibodies on interaction between VISTA-Fc-d2 and PSGL1-His.

Assay principle:

The HTRF (Homogeneous Time-Resolved Fluorescence) technology is an assay developed to study the interaction between biomolecules. This detection system is based on a fluorescence resonance energy transfer (FRET). The interaction between hVISTA-Fc labelled with d2 (VISTA-Fc-d2) and His-tagged hPSGL-1 (PSGL1-His) bearing a Terbium-labelled anti-His mAb (Anti His-Tb / Cisbio) allows the occurrence of a HTRF signal.

The antibodies tested in the experiment were:

- anti-VISTA Ab1
- anti-VISTA R&D Systems #71261
- human IgG1 control isotype.

The antibodies were incubated at different concentrations (0 to 20 µg/mL) during 4 hours at room temperature and at pH=6 with VISTA-Fc-d2 and PSGL1-His indirectly labelled with anti-His-Tb.

No diminution of signal is observed with the negative control. On the other hand, the addition of the positive control, *i.e.*, commercial antibody (R&D System #71261) preventing VISTA/PSGL-1 interaction, results in the decrease of the measured HTRF signal, as expected. The IC_{50} measured for this antibody was 333 nM (Table 8). Importantly, Ab1 also triggers a decrease of the signal, indicating the antibody blocks the interaction between VISTA and PSG-L1 at acidic pH (see Table 9). The IC_{50} of Ab1 in VISTA/PSGL-1 HTRF interaction assay was 2.3 nM (Table 7).

Table 8: Anti-VISTA antibodies IC_{50} in VISTA/PSGL-1 HTRF interaction assay

	IC_{50} (nM)
Anti-VISTA (R&D Sytems)	333
Anti-VISTA Ab1	2.3

Table 9: Mean of 3 experiments expressed in percentage of the specific HTRF signal

	Antibody concentration												
	0 µg/mL	0,01 µg/mL	0,02 µg/mL	0,04 µg/mL	0,08 µg/mL	0,16 µg/mL	0,31 µg/mL	0,63 µg/mL	1,25 µg/mL	2,5 µg/mL	5 µg/mL	10 µg/mL	20 µg/mL
hIgG1 (control isotype)	98	91	93	92	91	102	95	102	95	95	96	98	97
anti-VISTA (R&D Systems)	96	100	106	99	96	89	88	81	68	63	50	34	21
anti-VISTA Ab1	104	96	97	98	89	74	58	43	32	23	19	11	13

Example 6: *In vivo* evaluation of anti-VISTA mAb1 antibody in the MC38 murine colon tumour model

Materials and Method

For each experiment, a frozen vial of MC38 cells was thawed and grown in DMEM/F12 with 10% serum. After 2 days in culture, the cells were harvested using trypsin and resuspended in DMEM/F12 at a concentration of $5x10^5$ cells/ml and 100 µl injected per mouse.

Female C57Bl/6 hVISTA mice aged 8-10 weeks were purchased from Genoway (Lyon, France). Upon arrival they were allowed to acclimatise for 7 days prior having their right flanks shaved. Mice were injected subcutananeously (s.c.) on their shaved flank, with 100 µl of MC38 cell suspension (50,000 cells).

Tumours were considered established once they reached ~6mm in diameter (~80 mm^3 volume). Once established, treatment was initiated. Murinised anti-VISTA antibody (mAb1), corresponding to the CDRs of Ab1 with a murine Fc, or corresponding isotype control antibody mIgG2a were administrated intraperitoneally at 30mg/kg (formulated in Histidine 25 mM, NaCl 150mM, 0.1% Polysorbate 80 [v/v], pH 6.5), every 3 to 4 days for a total of 4 injections. Tumour growth was evaluated three times per week over the course of treatment and until the experiment was terminated, using electronic calipers across the three dimensions: length (L), width at a 90° angle to the first measurement (W) and finally height (H).

Tumour volume was derived as follows: Volume = 0.52x(LxWxH)

Because T-cell activation by Ab1 was shown in vitro to be dependent on the effector functions, the role of these activities in any anti-tumour activity of the antibody was investigated. A variant of mAb1 was created in which the Asn interacting with the FcγR (the equivalent residue of N298A) in Ab1) was replaced with an Ala residue, thereby eliminating any effector mechanism. A mIgG1 antibody was used as a negative control (Chen et al. *Front Immunol.* **10**:292 (2019).

Results

In the tested conditions of engraftment and schedule of administration, mAb1 in competent format induces a tumour growth inhibition of 47% at day 21 (Fig. 7). The silent format of mAb1 does not induce tumour growth inhibition (Fig. 8).

Example 7: Design of a formulation for Ab1

Formulation development, an important aspect of product development, is often on the critical path to successful clinical manufacturing and stability studies,

which are essential to investigational new drug (IND) filings. Antibodies have usually been administered by infusion due notably to their large size. Because of their complex three-dimensional structures, antibodies tend to aggregate in solution, thus decreasing their shelf-life and therefore their usability.

A pre-formulation study was performed to select four antibody formulations using a two steps approach based on experimental designs. The aim of this work was to define four antibody formulations using a two steps approach based on experimental designs. The first step was dedicated to important factors identification and the second one to four formulations definition.

Samples, stored at +5±3°C, +40°C or submitted to 3 Freeze/Thaw cycles, were analysed after different periods of time, from D0 to 4 weeks by UV280nm, size exclusion chromatography (SEC), Flow Field-Flow Fractionation (A4F), Thermal Shift Assay (TSA) or Differential Scanning Calorimetry (DSC), cation exchange (CEX) chromatography and particles amount by Micro-Flow Imaging (MFI).

7.1 Material & Methods

Experimental design

Design of step 1

The following factors were selected for this step:

- Buffer: 25 mM Citrate (C) or 25 mM Histidine (H) or 25 mM Phosphate (P)
- pH: 5.5 or 6 or 6.5
- Sucrose (Suc): 0 - 6% (mass/volume) (m/v)
- Arginine (Arg): 0 - 500 mM
- NaCl: 0 - 150 mM
- Surfactant: no Polysorbate (PS), Polysorbate 80 0.01% or Polysorbate 20 0.01% (volume of surfactant/volume of mAb solution) (v/v)

The mAb concentration was fixed to 20 mg/mL.

The experimental design was set up using the MODDE (Umetrics) software under the screening mode, which allows finding the important factors as the first phase of a project using linear and interactions models.

Design of step 2

The following factors have been selected for this step:

- Buffer: 25 mM Histidine
- pH: 5.5 - 6.5
- Sucrose: 0 - 6% (m/v)
- NaCl: 0 - 150 mM
- Polysorbate 80: 0 - 0.01% (v/v)

The mAb concentration was fixed to 20 mg/mL.

The experimental design was set up using the MODDE software under the optimisation mode (Response Surface Model, RSM), which allows detailed modelling and optimisation using quadratic and cubic models. The centre point has been analysed in triplicate to obtain an estimation of the experimental variability.

Protocol

For both steps, Ab1 at 20 mg/mL was formulated in the convenient buffer by overnight dialysis at +5±3°C. The dialysed solutions were centrifuged 2 min at 2000g prior mAb concentration determination by UV280 method. The concentration of the final formulations was adjusted by dilution with the corresponding buffer to 20 mg/mL. When required Polysorbate 20 or Polysorbate 80 were added (volume of Polysorbate /volume of mAb solution). After sterile filtration (0.22µm filters), sterile 2 mL Eppendorf tubes were filled with 0.3 mL of each formulation and stored as follow prior their analysis. Additionally, in step 2 sterile 15 mL Falcon tubes were filled with 2.5 mL of each formulation and stored 4 weeks at +40°C.

Table 10

		Step 1	Step 2
Storage conditions - Stress applied	+5±3°C +40°C 3 Freeze/Thaw cycles	10 days 10 days x	2 and 4 weeks 2 and 4 weeks x
Responses		mAb amount monomers content melting temperature (T0 only)	mAb amount monomers content melting temperature (T0 only) particles amount (T0 only and T4w_40°C)

Statistical analysis

Experiments were analysed using the MODDE special software.

Materials

Equipment

- NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific)
- MicroCal VP-Capillary DSC (Malvern Instruments) - software: MicroCal VP-Capillary DSC software 2.0
- HPLC systems:
  - Shimadzu LC10-AD - software LC Solution version 1.25
  - Agilent 1260 thermostat, UV detector 1260 (DAD) (Agilent)
  - Waters 2695 (Waters) - software Empower 2
- Eclipse Dualtec A4F separation system (Wyatt)
- A4F spacer Short-Channel (SC) 490 µm W, Regenerated Cellulose membrane 10 kDa
- Multi-angle light scattering (MALS) detector (Wyatt Dawn HELEOS II)
- RI detector Optilab T-rEX (Wyatt)
- Acquisition and processing softwares for A4F: Open Lab C.01.06 and Astra 6.1.6.5

Reagents

- L-Histidine (Sigma Aldrich - H8000)
- L Histidine Monohydrochloride monohydrate (Sigma Aldrich - H8125)
- NaCl (Merck-Millipore – 106400)
- Sodium Azide NaN3 (Sigma Aldrich - S2002)
- BSA (Pierce – 23029)
- CAPDSC 96 well plates (Malvern Instruments – WEL190010-10)
- CAPDSC 96 well plates cover (Malvern Instruments – WEL190020-010)
- ProPac WCX-10 column (4x250mm) (ThermoScientific – 054993)
- CX-1 pH gradient buffer A pH 5.6 10x (Thermo Scientific – 085346)
- CX-1 pH gradient buffer B pH 10.2 10x (Thermo Scientific – 085348)

- Carbonic anhydrase (Sigma Aldrich – C7025) prepared at 2 mg/mL in 25 mM His/His-HCl, 150 mM NaCl buffer pH 6.5
- Ab1

Methods of analysis

Table 11: Methods used in the study

Test	Method
mAb concentration	UV280
Aggregates, multimers, degradation products	SEC
Aggregates, multimers, degradation products	A4F
Melting temperature Tm	DSF/TSA
Melting temperature Tm	DSC
Charge variants	CEX
Particles >10 µm & >25 µm	MFI

Protein concentrations were determined by UV280 protein assay on NanoDrop One Spectrophotometer.

Aggregation and fragmentation were assessed by Asymmetric Flow Field Flow Fractionation (A4F). For each sample, 40 µg were injected onto a 490 µm W, short channel. IgG were eluted using 25 mM Histidine, 150 mM sodium chloride, pH 6.5 with 0.02% NaN_3. Samples were analysed by Size Exclusion Chromatography "SEC" on a Superdex 200 Increase 10/300GL column connected to 2695 Waters HPLC system.

Samples Melting Temperature (Tm) were determined by Differential Scanning Fluorimetry (DSF) using standard protocols.

Samples Melting Temperature (Tm) were also determined by Differential Scanning Calorimetry (DSC). Each sample was diluted in its own dialysis buffer to reach a protein concentration of 1mg/mL. 400µL of these 1 mg/mL solutions and 400 µL of each dialysis buffer (as blank run) were dispensed in 96 well plates. DSC analyses were performed in an automated microcalorimeter with a temperature range from 40 to 100°C and a scan rate of 60°C/h.

Charge variants were analysed by Cation Exchange Chromatography (CEX) on a ProPac WCX-10 column connected to Shimadzu LC10-AD HPLC system. 20 µg of each sample were injected on the column. 5 µg of carbonic anhydrase and 20 µg of hz1022C3 L1/H13 (G1) were also injected at the beginning of the batch as calibrants.

Running conditions: CX-1 pH gradient buffer A pH 5.6 1x and CX-1 pH buffer B pH 10.2 1x - flow: 1mL/min.

Results were analysed with MicroCal VP-Capillary DSC automated analysis software.

7.2 Results

Results of step 1

Thermal Shift Assay at T0

TSA analyses have been performed on the 22 conditions. The profile of the derivative plots as well as the Tm value change according to the buffer nature (Fig. 9). The triplicate on sample 11 shows good reproducibility of the experiment and the standard deviation indicates that a difference of 1°C is significant. The TSA results were statistically scrutinised (Fig. 10). Only 3 factors have been identified as important factors for each Tm. 25 mM Citrate buffer pH 5 and 500 mM Arginine have a negative effect on both Tm. 25 mM Phosphate buffer pH 6.5 and 6% sucrose have a positive effect on Tm1 and Tm2 respectively.

Remaining mAb Analysis

At each time point the Ab1 concentration was determined. The amount of remaining mAb is expressed in % of the initial amount. The results were statistically scrutinised (Fig. 11). 25 mM Phosphate buffer pH 6.5 and 500 mM Arginine have a negative effect on remaining mAb after 10 days at 40°C as well as 25 mM Citrate buffer pH 5.5 after the freeze/thaw cycles.

Monomers Analysis

SEC analyses have been performed on the 22 conditions for each time point, the monomers content is expressed in percentage. At T0 the monomer content for each condition is comparable. The triplicate on sample 11 shows good reproducibility of the experiment and the standard deviation indicates that a difference of 1% is significant. The results were statistically scrutinised (Fig. 12). Again 25 mM Phosphate buffer pH 6.5 has a negative effect on monomers after 10 days at 40°C and after the freeze/thaw cycles. Additionally, after 10 days at 40°C absence of detergent has

negative effect. 500 mM Arginine has a positive effect on monomers content after 10 days at 40°C.

Step 1 Results Overview

Table 12 below summarises the weight of each studied factor, calculated through MODDE, for the step 1.

Table 12:

	TSA		Remaining mAb (%)				Monomers (%)			
	Tm1	Tm2	T0	T10d_ 4°C	T10d_ 40°C	F/T x3	T0	T10d_ 4°C	T10d_ 40°C	F/T x3
C5	-5.4	-1.3	0	0	0	0	0	0	0	0
C5.5	0	0	0	0	0	-1.6	0	0	0	0
C6	0	0	0	0	0	0	0	0	0	0
H6	0	0	0	0	0	0	0	0	0	0
H6.5	0	0	0	0	0	0	0	0	0	0
P6	0	0	0	0	0	0	0	0	0	-0.76
P6.5	2.8	0	0	0	-0.6	0	0	0	-0.4	0
NaCl	0	0	0	0	0	0	0	0	0	0
Sucrose	0	0,4	0	0	0	0	0	0	0	0
Arginine	-1.4	-1.2	0	0	-0.3	0	0	0	0.4	0
PS20	0	0	0	0	0	0	0	0	0	0
PS80	0	0	0	0	0	0	0	0	0	0
no PS	0	0	0	0	0	0	0	0	-0.2	0

All the factors presenting a negative effect were not more considered for the next step.

The first step identified 25 mM Citrate buffer pH 5 or pH 5.5, 25 mM Phosphate buffer pH 6 or pH 6.5 and 500 mM Arginine as factors having negative effects on melting temperatures, remaining mAb and monomers content.

The selected factors to be further investigated were

- Histidine buffer pH 5.5 to 6.5

- NaCl: 0 -150 mM
- Sucrose: 0 - 6% (m/v)
- Polysorbate 80: 0 - 0.01% (v/v)

Results of step 2

Ab1 analyses at T0

Before being put under stress conditions, Ab1 was analysed. The mAb concentration was homogeneous between samples. The average percentage of monomers, SEC UV and A4F, as well as the percentage of the principal peak of the charge variants were similar for all the formulations. Therefore, at T0, buffer has a negligible influence on monomers and charge variants. However, particles amount at T0 varies from one buffer to another indicating a potential influence of the buffer on this parameter.

DSC analyses reveal different profiles and Tm values change according to the buffer nature.

Experimental design results

The results obtained after the stress of freeze-thaw cycles as well as those obtained after storage at +5±3°C, T2weeks and T4weeks, or after the stress at 40°C for 2 weeks were not exploited because the stress does not generate enough significant changes in the studied responses (percentage of remaining mAb, percentage of monomers by SEC UV or AF4 and percentage of principal charge variants peak).

The results obtained for the particles were not analysed because they looked inconsistent.

Complete results for the responses scrutinised by MODDE show that, for each of the studied response, the reproducibility of the triplicates is good. The analysis of all the responses obtained versus the responses predicted by the model shows that the regression is good, which means that the established model is a good model.

Response surface

Surface response analyses allowed to finely examine the behaviour of each response. It was thus observed that:

- A pH of 6.5 promotes high melting temperatures, but lowers the monomer level as well as the percentage of the main peak of charge variants.
- A high concentration of NaCl (150 mM) is not favourable for reaching optimal melting temperatures as well as high level of monomers.
- Overall, NaCl concentration greater than 80 mM is favourable for maintaining a good percentage of the main peak of charge variants.
- Sucrose as well as Polysorbate 80 have little effect on melting temperatures and level of monomers.
- However, 0.25% Polysorbate 80 (mass/mass) has a positive effect on the principal peak rate of charge variants.

Optimum points

Optimum points defined by the MODDE software are summarised in Table 13 below.

Table 13: Optimum points of step 2

		Histidine buffer pH	NaCl (mM)	Sucrose (% m/v)	PS80 (% v/v)
DSC	Tm#1 &#2	6.4	15	5.4	0.009
Forced degradation T4w_40°C	SEC UV	5.9	5.8	0.65	0.006
	A4F	6.2	150	5.8	0.01
	Variants	6.5	147	0.06	0.0054
	SEC UV_Variants	6.3	147	0.08	0.006
	A4F_Variants	6.5	150	6	0.0094

In the second step of the study the search for optimum points revealed that

- The most favourable pH range is between 5.9 and 6.5
- The most favourable NaCl concentration varies according to the studied response. For the melting temperature and for the monomers content (based on SEC UV) low concentration is required, but for charge variants and combined analysis of SEC UC_charge variant high concentration is required.
- The most favourable Sucrose concentration varies according to the studied response. For the melting temperature, for the monomers content based on A4F and for the combined analysis of A4F_charge

variants high concentration is required, but for SEC UV, charge variants and combined analysis of SEC UC_charge variant low concentration is required.

- The most favourable concentration of Polysorbate 80 is between 0.006 and 0.01% (v/v).

Based on these results, four formulations were selected for a stability study:

- A: 25 mM Histidine, 1 % sucrose, 0.006% Polysorbate 80 (v/v), pH 6;
- B: 25 mM Histidine, 150 mM NaCl, 0.006% Polysorbate 80 (v/v), pH 6.5;
- C: 25 mM Histidine, 150 mM NaCl, 3 % sucrose, 0.006% Polysorbate 80 (v/v), pH 6.5; and
- D: 25 mM Histidine, 15 mM NaCl, 5 % sucrose, 0.01% Polysorbate 80 (v/v), pH 6.5.

Example 8: Stability study of a formulation for Ab1

A stability study was performed with the 4 formulations selected in Example 7. The formulations were kept at -66°C, +5°C, and +40°C for 1.5 month and 2 months + 3 weeks. The following tests were performed: appearance, opalescence, pH, protein content by UV, SEC-HPLC, antibody purity by CE-SDS (non-reduced), charge profile by CEX, DSC, MFI and target binding by ELISA.

8.1 Material & Methods

Preparation of the formulations

The batch was re-formulated in each formulation (A, B, C and D) by running separate tangential flow filtrations (TFF). Each TFF was performed using Sius Hystream LS 30kDa 0.01 m^2 cassettes from Repligen. The product was processed at 300 LMH without back pressure. At the end, product concentration was adjusted at 20 g/L.

Polysorbate 80 was added at the end using a concentrated solution (5% v/v Polysorbate 80), just before 0.22µm filtration on PES membrane.

The antibody solutions were then dispatched in Pharmatainer 50mL tubes (PET reference 1000PF-05 from Cellon), filled with 4 or 7 mL/tube.

pH

The test is performed with pH meter in compliance with the European Pharmacopoeia chapter 2.2.3.

VISTA binding by ELISA EC_{50} ratio (in % of reference substance)

This test assesses direct binding between Ab1 solution and VISTA receptor coated onto a 96-well plate. After plate blocking, Ab1 solution is serially diluted across the plate to allow binding with the receptor. The complex is detected with goat anti-human IgG, conjugated to Horseradish Peroxidase (HRP), and quantified using a colorimetric substrate. Four-parameter logistic fits are used for data analysis to derive EC_{50} values for Ab1 solution which is compared to the reference EC_{50} value.

Charge profile by CEX (%)

Charge variants heterogeneity of Ab1 solution were studied by a method based on liquid ionic exchange chromatography and developed according to Ph. Eur. 2.2.29. The sample is desalted and diluted at about 5 mg/mL in water and analysed as described below:

Table 14

Detector	UV detector λ = 280 nm			
Column	MabPac SCX-10: 4 x 250 mm, 10 µm (or equivalent) Temperature: 30°C			
Mobile Phase	A: CX-1 pH Gradient Buffer A pH = 5.6 B: CX-1 pH Gradient Buffer B pH = 10.2			
Gradient	Time (min)	%A	%B	pH
	0	100	0	5.6
	1	100	0	5.6
	18	63	37	7.3
	21	63	37	7.3
	21.5	0	100	10.2
	23.5	0	100	10.2
	24	100	0	5.6
	34	100	0	5.6
Flow Rate	0.6 mL/min			

Injection Volume	10 µL			

The quantification is achieved by normalisation procedure. Results for acidic variant peaks, main peak and basic variant peaks are reported as relative % of total area.

Size variants by SEC (%)

To study size variants of the Ab1 solution, a method based on Liquid Chromatography (Ph. Eur. 2.2.29) was developed according to the conditions below:

Table 15

Detector	UV detector λ = 280 nm
Column	BEH 200 UPLC 4.6 x 150 mm, 200Å and 4.6 x 30 mm (or equivalent) Temperature: 40°C
Mobile Phase	A: 100M sodium phosphate buffer/150mM NaCl, pH7.5
Isocratic mode	Run: 10 min.
Flow Rate	0.3 mL/min
Injection Volume	10 µL

The sample is diluted at about 1.5 mg/mL in mobile phase. The quantification is achieved by normalisation procedure. Results for aggregates, monomer and fragments peaks are reported as relative % of total area.

Antibody purity by CE-SDS (% of H2L2)

SDS gel capillary electrophoresis (CE-SDS) was used to evaluate Ab1 solution samples purity and integrity. Measurements were carried out using a Beckman-Coulter ProteomeLab PA800 equipped with a Photodiode array detector (220 nm). For the non-reduced conditions, the sample was diluted with the SDS-MW sample buffer. The internal standard and iodoacetamide (alkylating agent) were added to the solution and heated at 70°C for 10 minutes. For the reduced conditions, the sample was diluted with the SDS-MW (denaturing agent) sample buffer. The internal standard and 2-

mercaptoethanol (reducing agent) were added to the solution and heated at 70°C for 10 minutes. The quantification is achieved by normalisation procedure (%).

Subvisible particles by light obscuration (Ph. Eur. 2.9.19)

This test is performed in compliance with the European Pharmacopoeia chapter 2.9.19. Method 1 (Light obscuration particle count test).

Protein content by UV (mg/mL)

Protein concentration in Ab1 solution samples is determined by UV spectrophotometry at 280 nm. Sample is diluted with water in the working range of 0.054 mg/mL to 0.705 mg/mL. The protein concentration is calculated dividing the absorbance reading by the determined Ab1 solution specific absorbance coefficient (1.45 $mL.mg^{-1}.cm^{-1}$). The value is then corrected with the dilution factor.

Bacterial Endotoxins (IU/mg)

This test is performed in compliance with the European Pharmacopoeia chapter 2.6.14 by kinetic chromogenic method.

Sterility

This test is performed in compliance with the European Pharmacopoeia chapter 2.6.1.

8.2 Results

After 2 months 3 weeks stability study the analytical criteria for antibody quality were not discriminatory.

Appearance, opalescence and pH of the formulations remained stable over the studied period.

No significant evolution of protein content by UV was observed, regardless of the temperature (<-66°C, +5°C or +40°C) and the formulation. The concentrations were maintained in the range of ± 10% of initial concentration.

SEC profiles were really stable and similar for the 4 frozen formulations (<-66°C). At +5°C, some variability was observed over time without significant differences between the 4 buffers. In accelerated conditions at +40°C, some

fragments appeared, thus decreased monomers level after 2 months 3 weeks storage. However, all formulations evolved in the same way. In conclusion, SEC profile could not be a discriminatory factor between the 4 formulations, but it could help in choosing storage temperature (+5°C seemed to be better for SEC profile stability).

CE-SDS profile was thoroughly studied because of sensitivity of the antibody to oxidation during the production process. Level of full IgG (H2L2) was totally stable at +5°C during 2 months 3 weeks. When frozen at <-66°C, decreasing levels of full IgG (87-88% at T0, down to 84-86% at T3 months) were observed for all formulations. The same trend was observed in accelerated conditions at +40°C. In conclusion, CE-SDS profile could not be a discriminatory factor between the 4 formulations, but it was discriminatory for storage temperature: freezing at <-66°C must be avoided. Lack of control of the freezing and thawing process (static process) could explain these trends.

CEX profile was unchanged overtime and no difference between formulations was observed, regardless of storage temperature.

No significant difference was observed for ELISA binding and particles quantitation by MFI overtime and within the four formulations.

Inasmuch as the analytical criteria for antibody quality were not discriminatory, the osmolarity of the buffer was taken into account.

Buffer A was hypotonic and on the contrary, buffer D was hypertonic. These 2 formulations were then ruled out. Between formulations B and C, formulation B - *i.e.* 25 mM Histidine, 150 mM NaCl, 0.006% Polysorbate 80 v/v), pH 6.5 - was chosen in order to limit the number of raw materials in the composition (especially to avoid sucrose which may contribute to endotoxins level).

8.3 Results of stability study of formulation B up to 48 months

The stability of the formulation buffer was checked through its physico-chemical parameters determined in long term condition +5°C +/- 3°C: appearance including opalescence, pH and osmolality. The stability data of formulation B at 48 months are shown in Table 16.

Conclusion of the study: the properties of the formulation buffer remained stable for at least 48 months at + 5°C.

Table 16: 48 months stability data of Ab1

Time points		T0	T1M	T2M	T3M	T6M	T9M	T12M	T18M	T24M	T36M	T48M
pH		6.6	6.4	6.4	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
VISTA binding by ELISA EC50 ratio (in % of reference substance)		115	95	88	100	88	109	94	87	83	91	101
Charge profile by CEX (%)	Main peak	72	NP	NP	72	73	NP	71	NP	72	69	66
	Acidic variants	20	NP	NP	20	20	NP	20	NP	24	25	27
	Basic variants	8	NP	NP	8	7	NP	8	NP	5	6	6
Size variants by SEC (%)	Monomer	99	99	100	100	99	99	99	99	99	98	97
	Aggregates	1	<1	< 1	< 1	1	1	1	1	1	1	1
	Fragments	ND	< 1	ND	ND	< 1	1	<1	<1	<1	1	2
Antibody purity by CE-SDS (% of H2L2)		97	95	97	96	94	95	96	95	96	96	96
Subvisible particles by light obscuration (Ph. Eur. 2.9.19)	≥ 10 µm (particles/vial)	702	NP	NP	24	17	NP	11	NP	15	8	28
	≥ 25 µm (particles/vial)	16	NP	NP	2	0	NP	2	NP	0	4	0
Protein content by UV (mg/mL)		22	22	22	22	22	22	21	21	21	21	21
Bacterial Endotoxins (IU/mg)		< 0.17	NP	NP	NP	NP	NP	< 0.17	NP	< 0.17	< 0.17	< 0.17
Sterility		Sterile	NP	NP	NP	NP	NP	Sterile	NP	Sterile	Sterile	Sterile

CEX = Cationic Exchange Chromatography ; CE-SDS = Capillary Electrophoresis – Sodium Docedecyl Sulfate; H2L2 = 2 Heavy Chains and 2 lights Chains; IU = International Unit; NP = Non Performed; SEC = Size Exclusion Chromatography ; UV = Ultra-Violet

CLAIMS

1. A pharmaceutical composition comprising:

   - a monoclonal anti-VISTA antibody comprising:
     - a heavy chain of sequence represented by SEQ ID NO:21 and
     - a light chain of sequence represented by SEQ ID NO:22,

   and

   - a pharmaceutically acceptable carrier and/or excipient.

2. The pharmaceutical composition of **claim 1**, wherein the concentration of the monoclonal anti-VISTA antibody is comprised between 5 and 100 mg/mL, preferably between 10 and 70 mg/mL, more preferably between 15 and 50 mg/mL, most preferably between 20 and 30 mg/mL.

3. The pharmaceutical composition of any one of **claim 1** or **claim 2**, wherein the concentration of the monoclonal anti-VISTA antibody is 20 mg/mL.

4. The pharmaceutical composition of any one of **claim 1** to **claim 3**, comprising a buffering agent, preferably a citrate buffer, a phosphate buffer, or a histidine buffer, more preferably a histidine buffer.

5. The pharmaceutical composition of **claim 4**, wherein the buffering agent is a histidine buffer at a concentration comprised between 5 and 45 mM, preferably 10 and 40 mM, more preferably 15 and 35 mM, most preferably 20 and 30 mM.

6. The pharmaceutical composition of any one of **claim 4** or **claim 5**, wherein the buffering agent is a histidine buffer at a concentration of 25 mM.

7. The pharmaceutical composition of any one of **claim 1** to **claim 6**, comprising a tonicity modifier, the tonicity modifier being preferably selected in the group consisting of polyhydric sugar alcohols, for example trihydric or higher sugar alcohols, such as glycerine, erythritol, arabitol, xylitol, sorbitol and mannitol, salts and amino acids; more preferably, a salt selected in the group consisting of sodium chloride, sodium succinate, sodium sulphate, potassium chloride, magnesium chloride, magnesium sulphate, and calcium chloride; even more preferably NaCl, $MgCl_2$, and/or $CaCl_2$, most preferably NaCl.

8. The pharmaceutical composition of **claim 4**, wherein the tonicity modifier is NaCl at a concentration comprised between 0 mM and 300 mM, preferably between 50 mM and 250 mM, more preferably between 100 mM and 200 mM NaCl.

9. The pharmaceutical composition of any one of **claim 7** to **claim 8**, wherein the tonicity modifier is NaCl at a concentration of 150 mM NaCl.

10. The pharmaceutical composition of any one of **claim 1** to **claim 9**, comprising a non-ionic surfactant, preferably a polysorbate, such as Polysorbate 20 or Polysorbate 80, more preferably Polysorbate 80.

11. The pharmaceutical composition of **claim 10**, wherein the non-ionic surfactant is Polysorbate 80 at a concentration between about 0.001% and about 0.5% (v/v), between about 0.002% and about 0.2% (v/v), between about 0.003% and about 0.1% (v/v), between about 0.004% and about 0.009% (v/v), between about 0.005% and about 0.008% (v/v), or between about 0.006% and about 0.007%. (v/v).

12. The pharmaceutical composition of any one of **claim 10** to **claim 11**, wherein the non-ionic surfactant is Polysorbate 80 at a concentration of 0.006% (v/v).

13. The pharmaceutical composition of any one of **claim 1** to **claim 12**, wherein the pH of the pharmaceutical composition is comprised between about 3.0 and about 9.0, between about 4.0 and about 8.0, between about 5.0 and about 8.0, between about 5.0 and about 7.0, between about 5.0 and about 6.5, between about 5.5 and about 8.0, between about 5.5 and about 7.0, or between about 5.5 and about 6.5.

14. The pharmaceutical composition of **claim 13**, wherein the pH of the pharmaceutical composition is about 6.5.

15. The pharmaceutical composition of any one of **claim 1** to **claim 14** comprising 25 mM Histidine, 150 mM NaCl, 0.006% Polysorbate 80 (v/v), pH 6.5.

16. The pharmaceutical composition of any one of **claims 1 to 15**, for use in the treatment of a cancer, in a patient.

17. The pharmaceutical composition of any one of **claims 1 to 15**, for the use of **claim 16**, wherein the use comprises inducing an immune response in the patient.

18. The pharmaceutical composition of any one of **claims 1 to 15**, for the use of **claim 17**, wherein the immune response includes induction of $CD4^+$ T cell proliferation, induction of $CD8^+$ T cell proliferation, induction of $CD4^+$ T cell cytokine production, and induction of $CD8^+$ T cell cytokine production.

19. The pharmaceutical composition of any one of **claims 1 to 15**, for the use of any one of **claims 16 to 18**, wherein the cancer is selected from bladder cancer, breast cancer, cervical cancer, colon cancer, endometrial cancer, oesophageal cancer, fallopian tube cancer, gall bladder cancer, gastrointestinal cancer, head-and-neck cancer, haematological cancer (e.g., leukaemia, lymphoma, or myeloma), laryngeal cancer, liver cancer, lung cancer, lymphoma, melanoma, mesothelioma, ovarian cancer, primary peritoneal cancer, salivary gland cancer, sarcoma, stomach cancer, thyroid cancer, pancreatic cancer, renal cell carcinoma, glioblastoma, and prostate cancer.

20. The pharmaceutical composition of any one of **claims 1 to 15**, for the use of any one of **claims 16 to 19**, wherein the use comprises activation of the effector functions of the antibody.

21. The pharmaceutical composition of any one of **claims 1 to 15**, for the use of any one of **claims 16 to 20**, further comprising the administration of a second therapeutic agent.

22. The pharmaceutical composition of any one of **claims 1 to 15**, for the use of **claim 21**, wherein the second therapeutic agent is an anti-PD-1 antibody or an anti-PD-L1 antibody.

Fig. 1

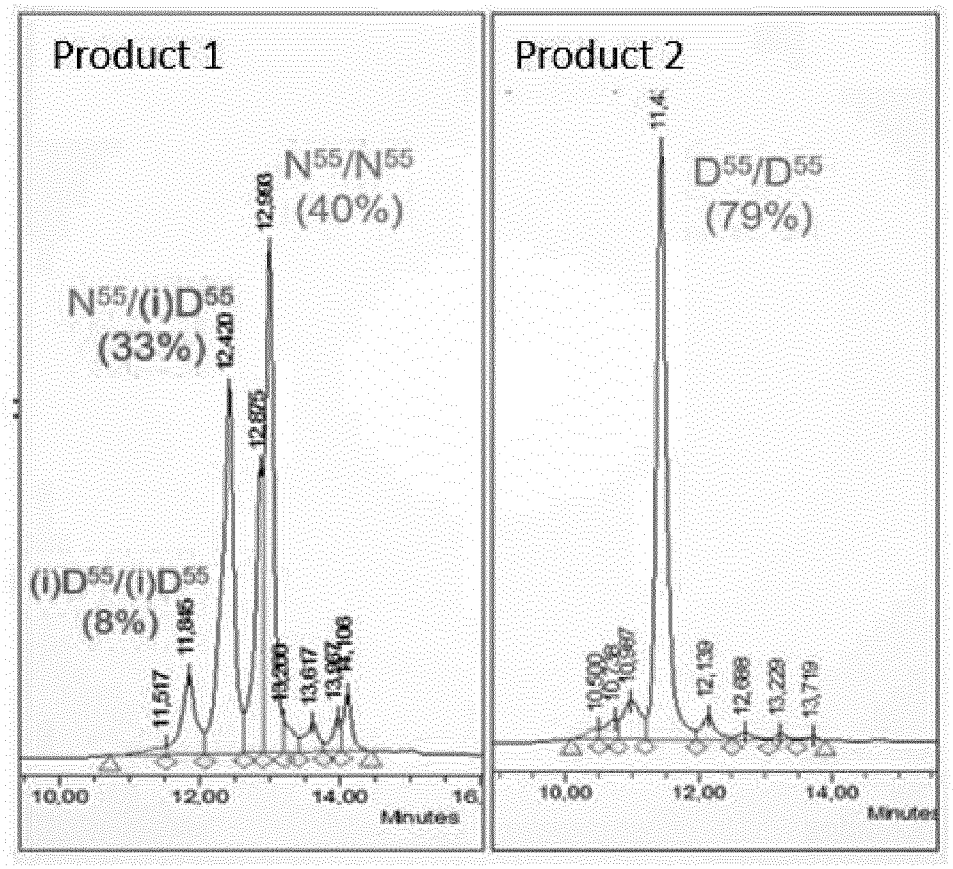

Product 1
N55/N55
(40%)
N55/(i)D55
(33%)
(i)D55/(i)D55
(8%)
12,563
12,430
12,875
11,517
11,845
13,617
10,00
12,00
14,00
Minutes
Product 2
D55/D55
(79%)
11,4
10,503
10,747
10,947
12,139
12,688
13,229
13,719
10,00
12,00
14,00

Fig. 2

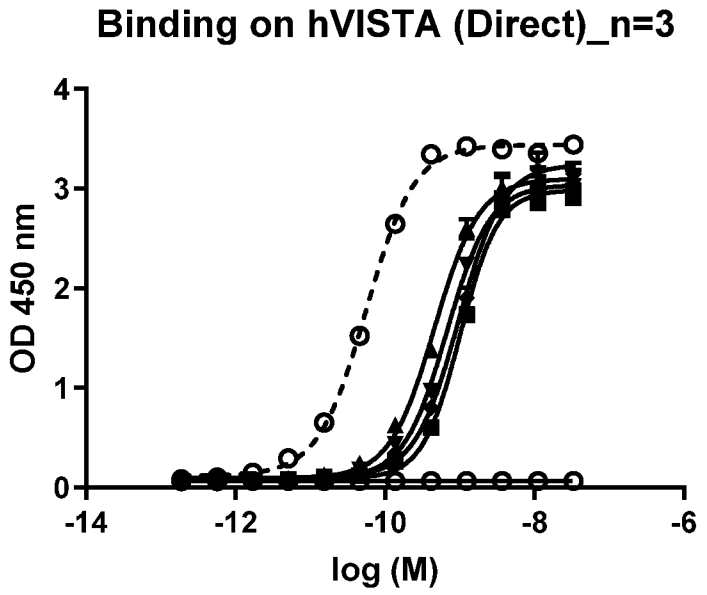

Binding on hVISTA (Direct)_n=3
OD 450 nm
0
1
2
3
4
-14
-12
-10
-8
-6
log (M)

Fig. 3

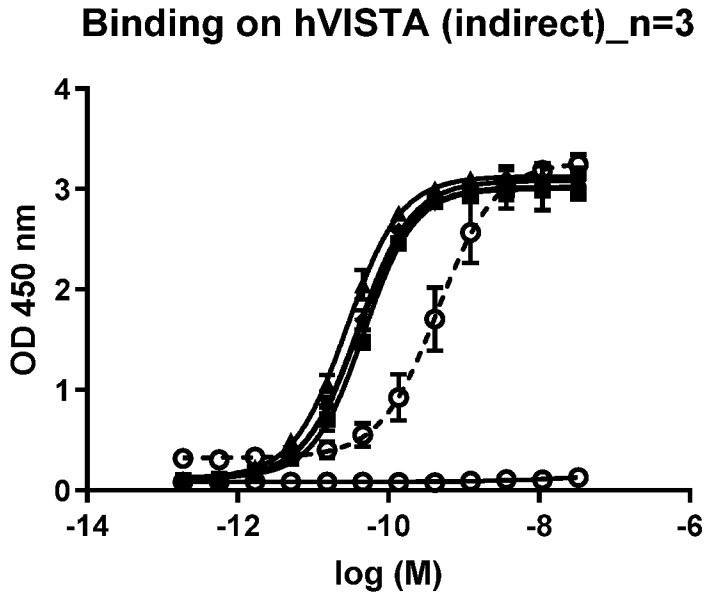

Binding on hVISTA (indirect)_n=3
OD 450 nm
0
1
2
3
4
-14
-12
-10
-8
-6
log (M)

Fig. 4

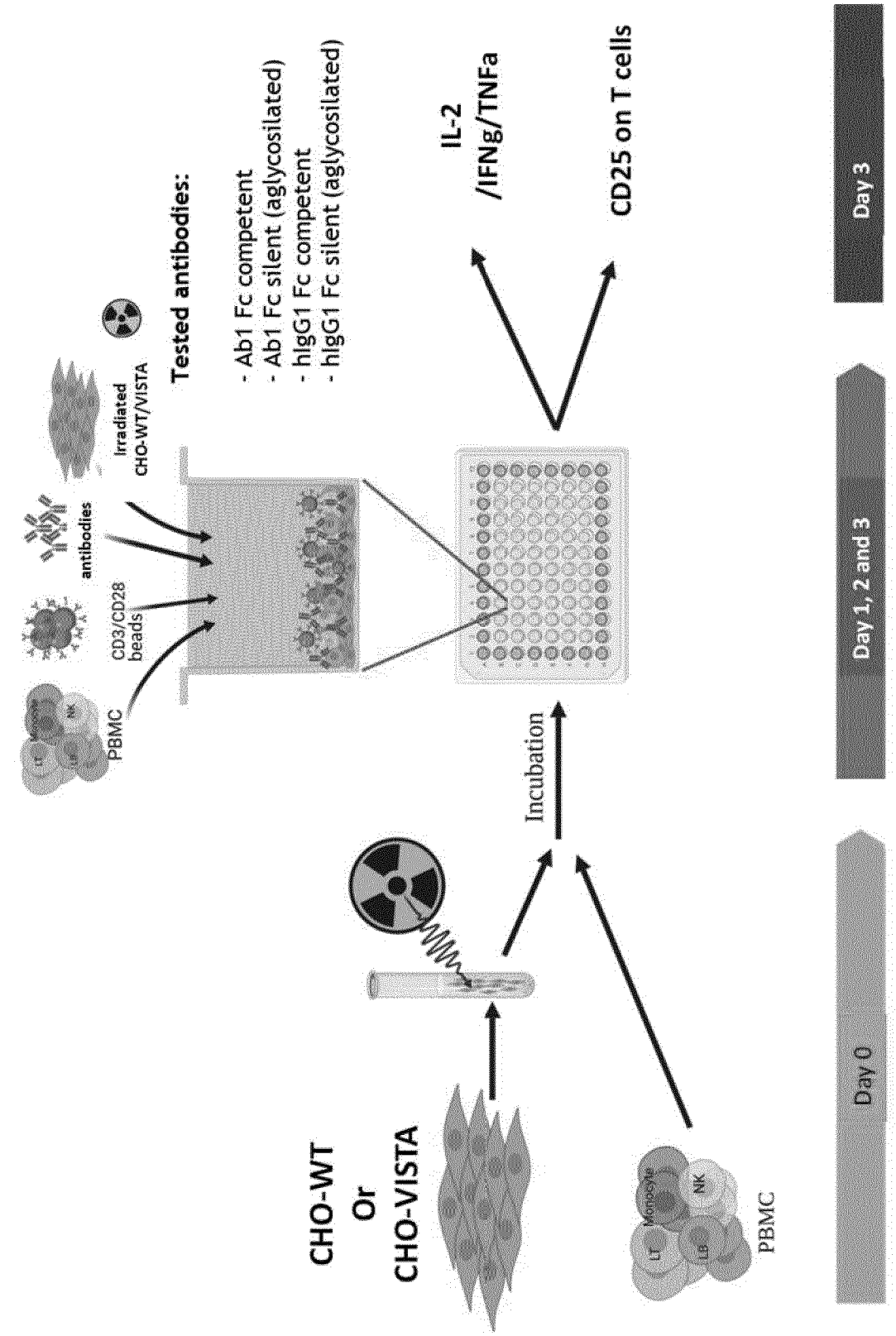

Day 0
Day 1, 2 and 3
Day 3
CHO-WT
Or
CHO-VISTA
PBMC
Incubation
IL-2
/IFNg/TNFa
CD25 on T cells
Tested antibodies:
- Ab1 Fc competent
- Ab1 Fc silent (aglycosilated)
- hIgG1 Fc competent
- hIgG1 Fc silent (aglycosilated)
Irradiated
CHO-WT/VISTA
antibodies
CD3/CD28
beads
PBMC

Fig. 5

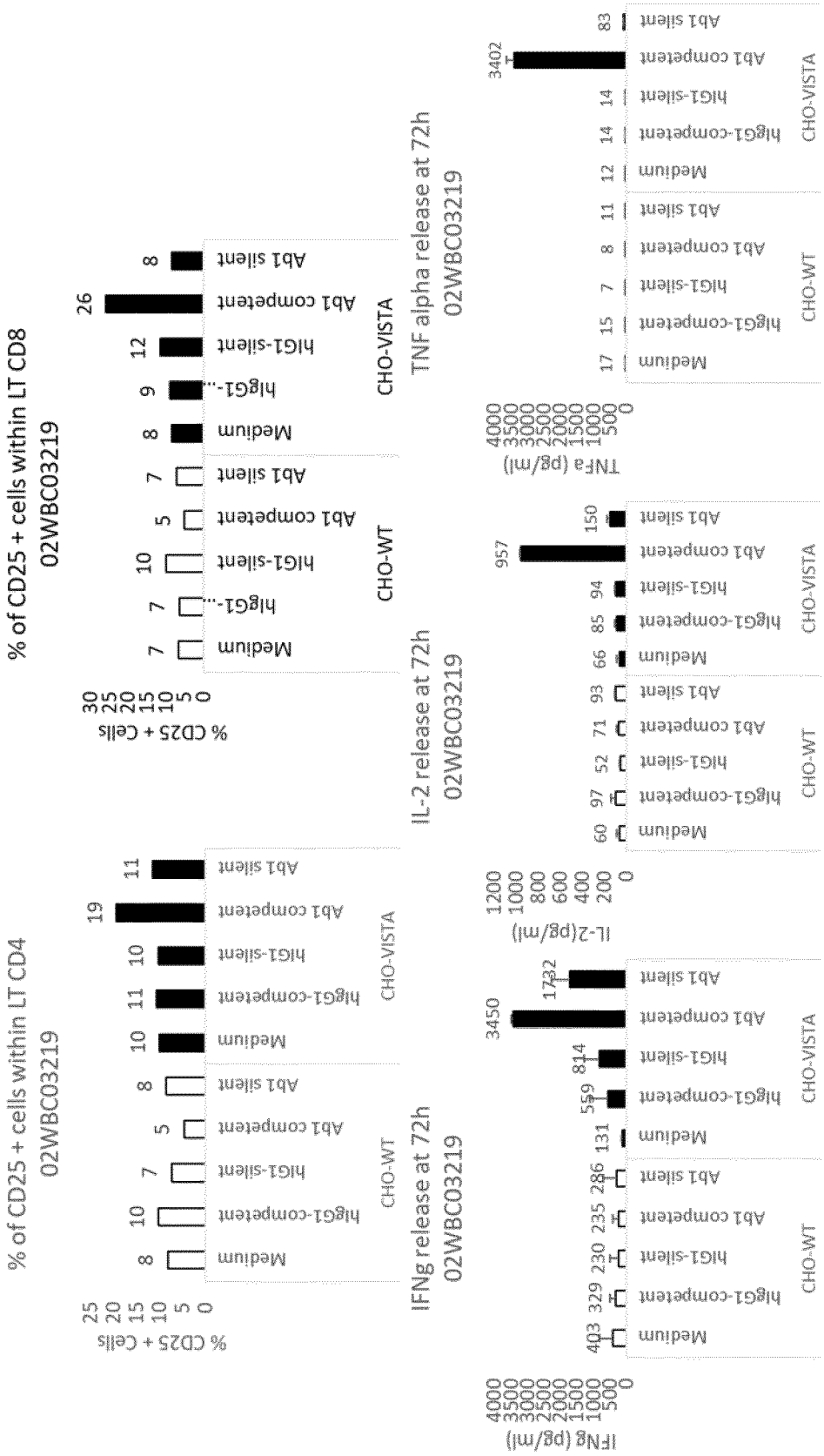

% of CD25 + cells within LT CD8
02WBC03219
% CD25 + Cells
CHO-WT
CHO-VISTA
Medium
hIgG1-competent
hIgG1-silent
Ab1 competent
Ab1 silent
IL-2 release at 72h
02WBC03219
IL-2(pg/ml)
TNF alpha release at 72h
02WBC03219
TNFa (pg/ml)
% of CD25 + cells within LT CD4
02WBC03219
IFNg release at 72h
02WBC03219
IFNg (pg/ml)

Fig. 6

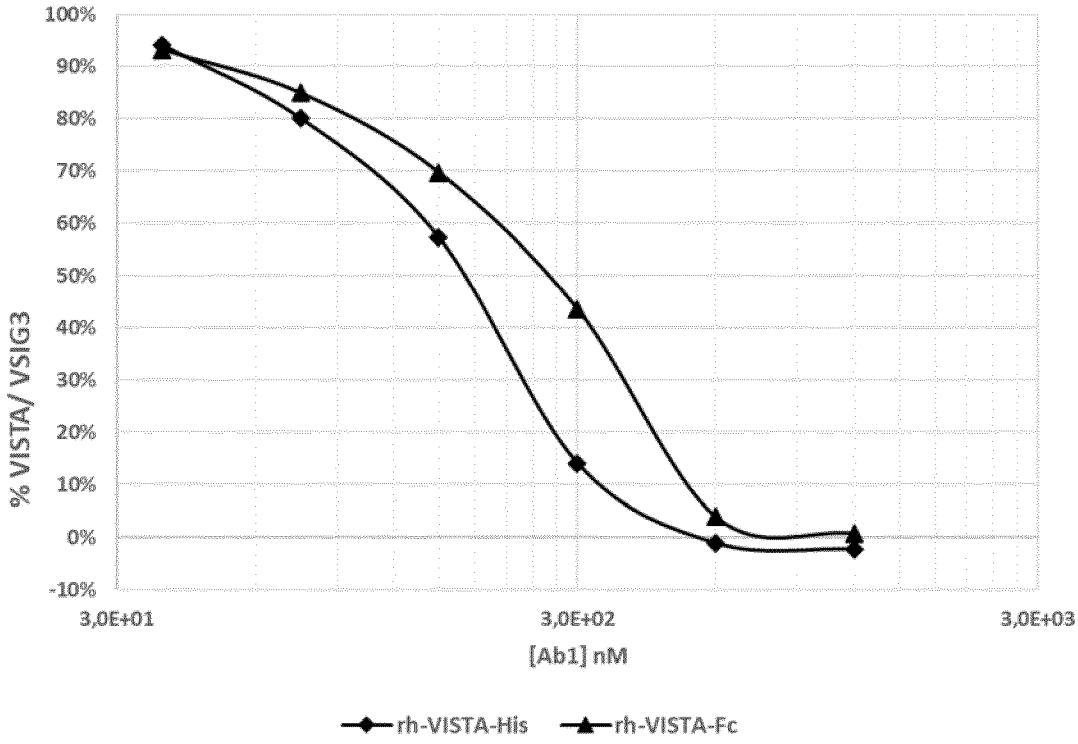

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
% VISTA/ VSIG3
3,0E+01
3,0E+02
3,0E+03
[Ab1] nM
rh-VISTA-His
rh-VISTA-Fc

Fig. 7

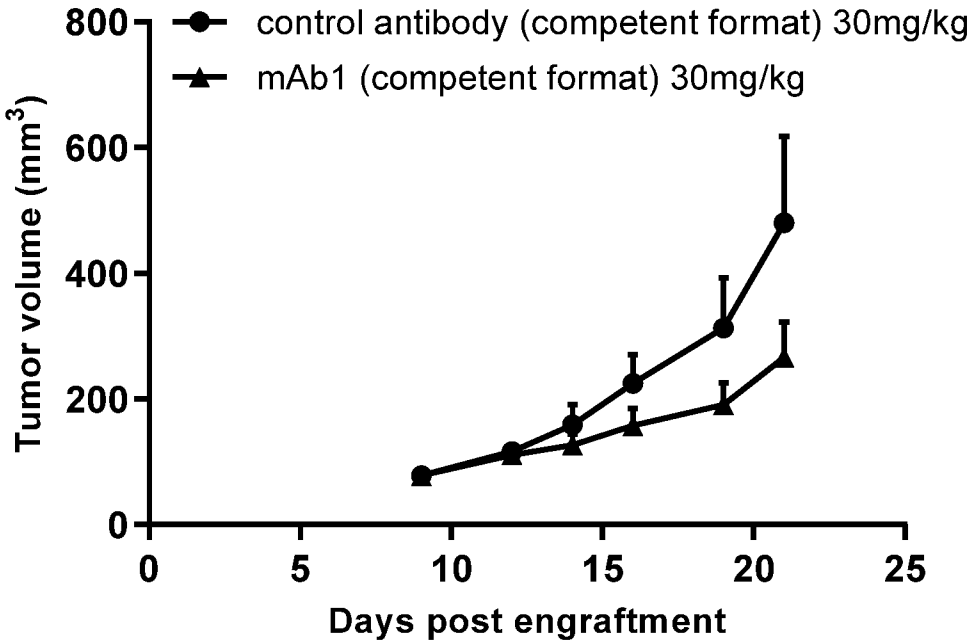

control antibody (competent format) 30mg/kg
mAb1 (competent format) 30mg/kg
Tumor volume (mm3)
800
600
400
200
0
0
5
10
15
20
25
Days post engraftment

Fig. 8

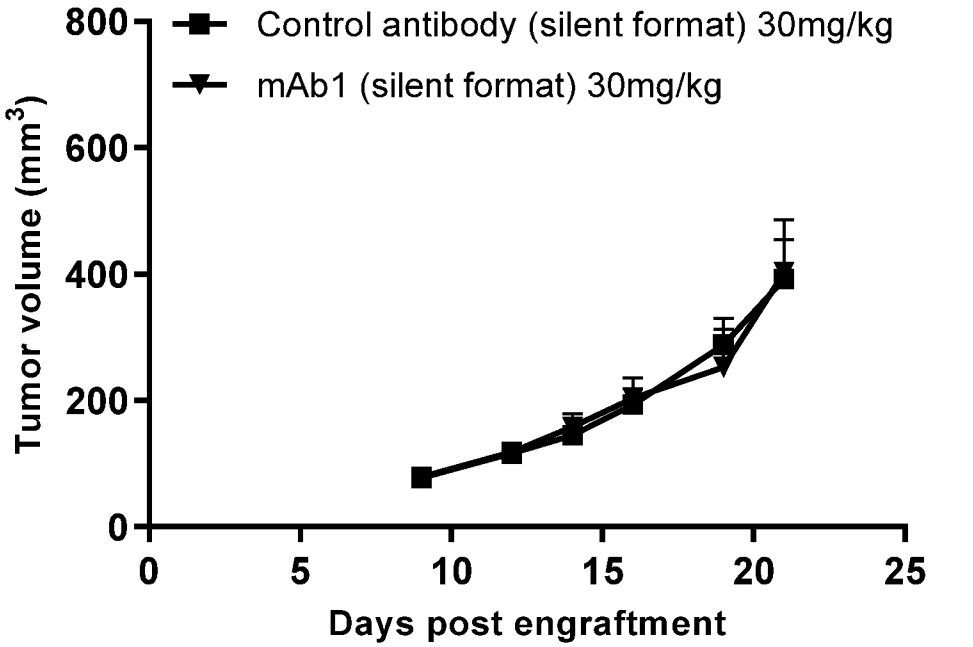
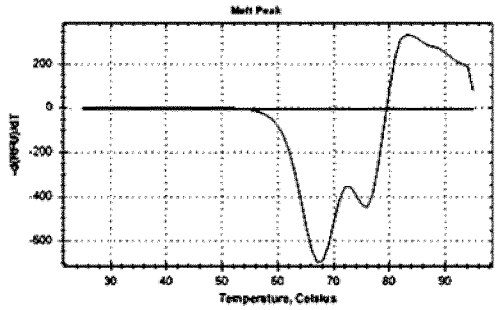

Control antibody (silent format) 30mg/kg
mAb1 (silent format) 30mg/kg
Tumor volume (mm3)
800
600
400
200
0
0
5
10
15
20
25
Days post engraftment

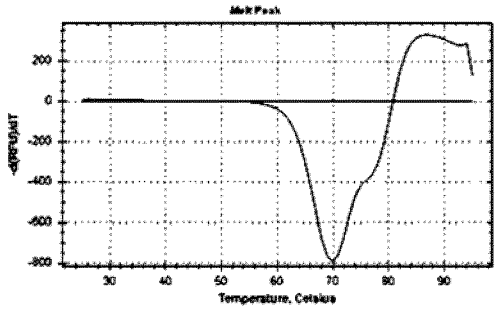
Fig. 9

A)

Conditions 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20,& 21



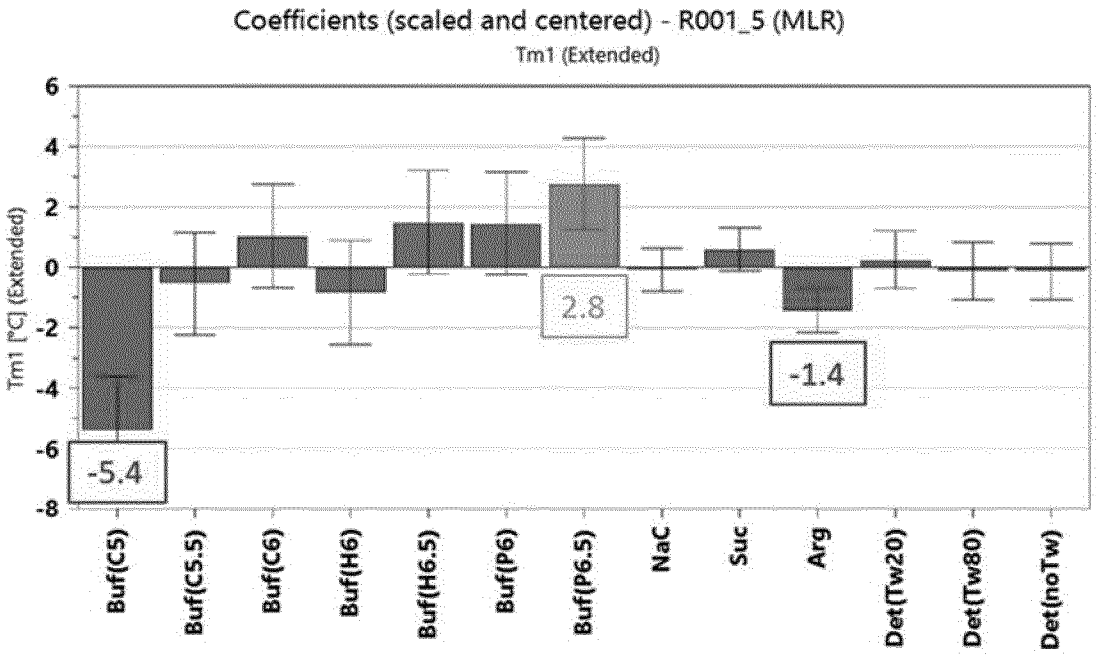
Conditions 5, 14 & 22



B)

		Tm1 (°C)					Tm2 (°C)				
		n=1	n=2	n=3	Mean value	SD	n=1	n=2	n=3	Mean value	SD
1	C5 Nac Suc Arg Tw20	61	61	61	61	0	73	73	73	73	0,0
2	C5.5 Arg Tw20	63	63	62	62.7	0.58	73	73	73	73	0,0
3	C6 Nac Tw20	67	67	67	67	0,0	76	75	75	75,3	0.58
4	H6 Nac Tw20	64	64	64	64	0,0	75	74	74	74,3	0.58
5	H6.5 Suc Tw20	70	71	70	70.3	0.58	76	75	76	75,7	0.58
6	P6 Suc Arg Tw20	65	65	65	65	0,0	74	74	74	74	0,0
7	P6.5 Nac Arg Tw20	65	66	66	65.7	0.58	72	73	73	72,7	0.58
8	C5 Suc Tw80	60	61	61	60.7	0.58	73	74	74	73,7	0.58
9	C5.5 Nac Arg Tw80	63	63	63	63	0,0	73	73	72	72,7	0.58
10	C6 Suc Arg Tw80	65	65	66	65.3	0.58	73	74	74	73,7	0.58
11a	**H6 Tw80**	**67**	**67**	**67**	**67**	0,0	**76**	**76**	**76**	**76**	0,0
11b	**H6 Tw80**	**66**	**66**	**66**	**66**	0,0	**76**	**75**	**76**	**75,7**	0.58
11c	**H6 Tw80**	**67**	**67**	**67**	**67**	0,0	**76**	**75**	**76**	**75,7**	0.58
12	H6.5 Nac Arg Tw80	64	63	64	63.7	0.57	73	71	73	72,3	0.58
13	P6 Nac Arg Tw80	65	64	65	64.7	0.58	73	73	73	73	0,0
14	P6.5 Nac Suc Tw80	69	69	69	69	0,0	76	75	76	75,7	0.58
15	C5	59	60	60	59.7	0.58	74	74	73	73,7	0.58
16	C5.5 Nac Suc	66	66	66	66	0,0	76	75	76	75,7	0.58
17	C6 Arg	65	64	64	64.3	0.58	73	73	73	73	0,0
18	H6 Nac Suc Arg	63	63	63	63	0,0	73	73	73	73	0,0
19	H6.5 Nac Suc Arg	65	65	65	65	0,0	73	74	73	73,3	0.58
20	P6 Nac	68	68	68	68	0,0	76	-	76	76	0,0
21	P6.5 Arg	65	65	65	65	0,0	72	73	73	72,7	0.58
22	P6.5 Nac Suc Arg	69	70	70	69.7	0.58	76	75	76	75,7	0.58
Samples 11					**66.7**	**0.58**				**75.8**	**0.19**

Fig. 10


Coefficients (scaled and centered) - R001_5 (MLR)
Tm1 (Extended)
Tm1 [°C] (Extended)
6
4
2
0
-2
-4
-6
-8
-5.4
2.8
-1.4
Buf(C5)
Buf(C5.5)
Buf(C6)
Buf(H6)
Buf(H6.5)
Buf(P6)
Buf(P6.5)
NaC
Suc
Arg
Det(Tw20)
Det(Tw80)
Det(noTw)

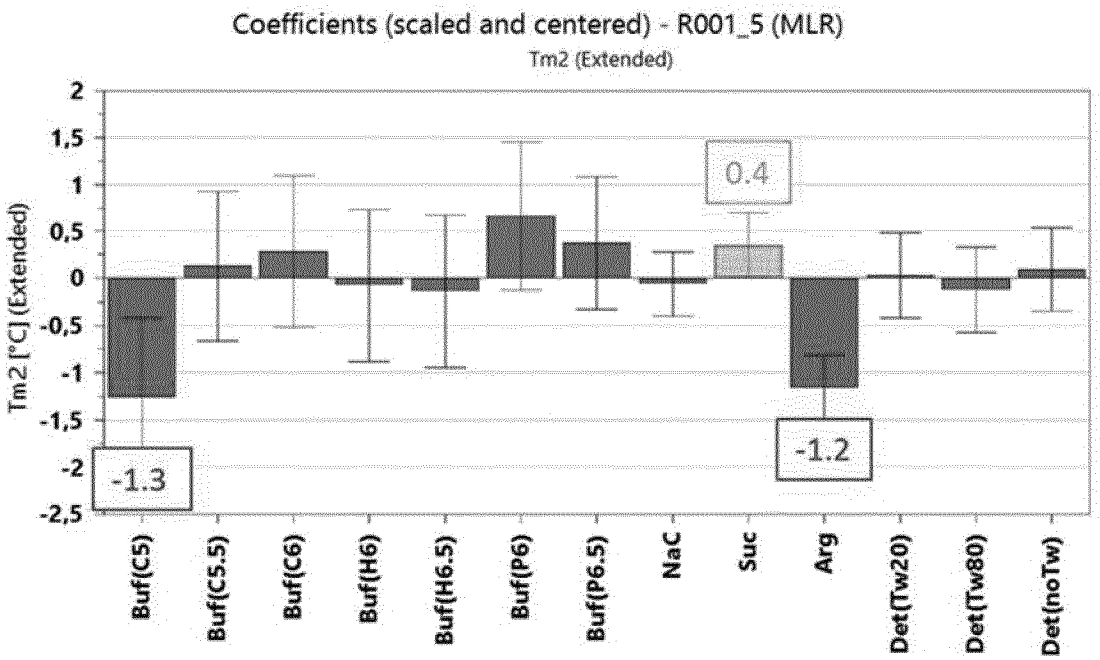

Coefficients (scaled and centered) - R001_5 (MLR)
Tm2 (Extended)
Tm2 [°C] (Extended)
2
1,5
1
0,5
0
-0,5
-1
-1,5
-2
-2,5
-1.3
0.4
-1.2
Buf(C5)
Buf(C5.5)
Buf(C6)
Buf(H6)
Buf(H6.5)
Buf(P6)
Buf(P6.5)
NaC
Suc
Arg
Det(Tw20)
Det(Tw80)
Det(noTw)

Fig. 11

A)

		T0	T10d_40°C	T10d_4°C	F/Tx3
1	C5 Nac Suc Arg Tw20	100	101	99	100
2	C5.5 Arg Tw20	100	101	100	99
3	C6 Nac Tw20	100	101	100	99
4	H6 Nac Tw20	100	102	100	100
5	H6.5 Suc Tw20	100	102	100	99
6	P6 Suc Arg Tw20	100	101	99	100
7	P6.5 Nac Arg Tw20	100	100	98	99
8	C5 Suc Tw80	100	101	99	99
9	C5.5 Nac Arg Tw80	100	102	94	95
10	C6 Suc Arg Tw80	100	101	99	100
11	H6 Tw80	100	102	100	100
12	H6.5 Nac Arg Tw80	100	101	100	100
13	P6 Nac Arg Tw80	100	101	100	101
14	P6.5 Nac Suc Tw80	100	101	101	100
15	C5	100	102	100	100
16	C5.5 Nac Suc	100	102	100	100
17	C6 Arg	100	101	100	100
18	H6 Nac Suc Arg	100	101	100	99
19	H6.5 Nac Suc Arg	100	102	100	101
20	P6 Nac	100	102	100	100
21	P6.5 Arg	100	101	100	99
22	P6.5 Nac Suc Arg	100	101	102	101

Fig.11 (continued)

B)

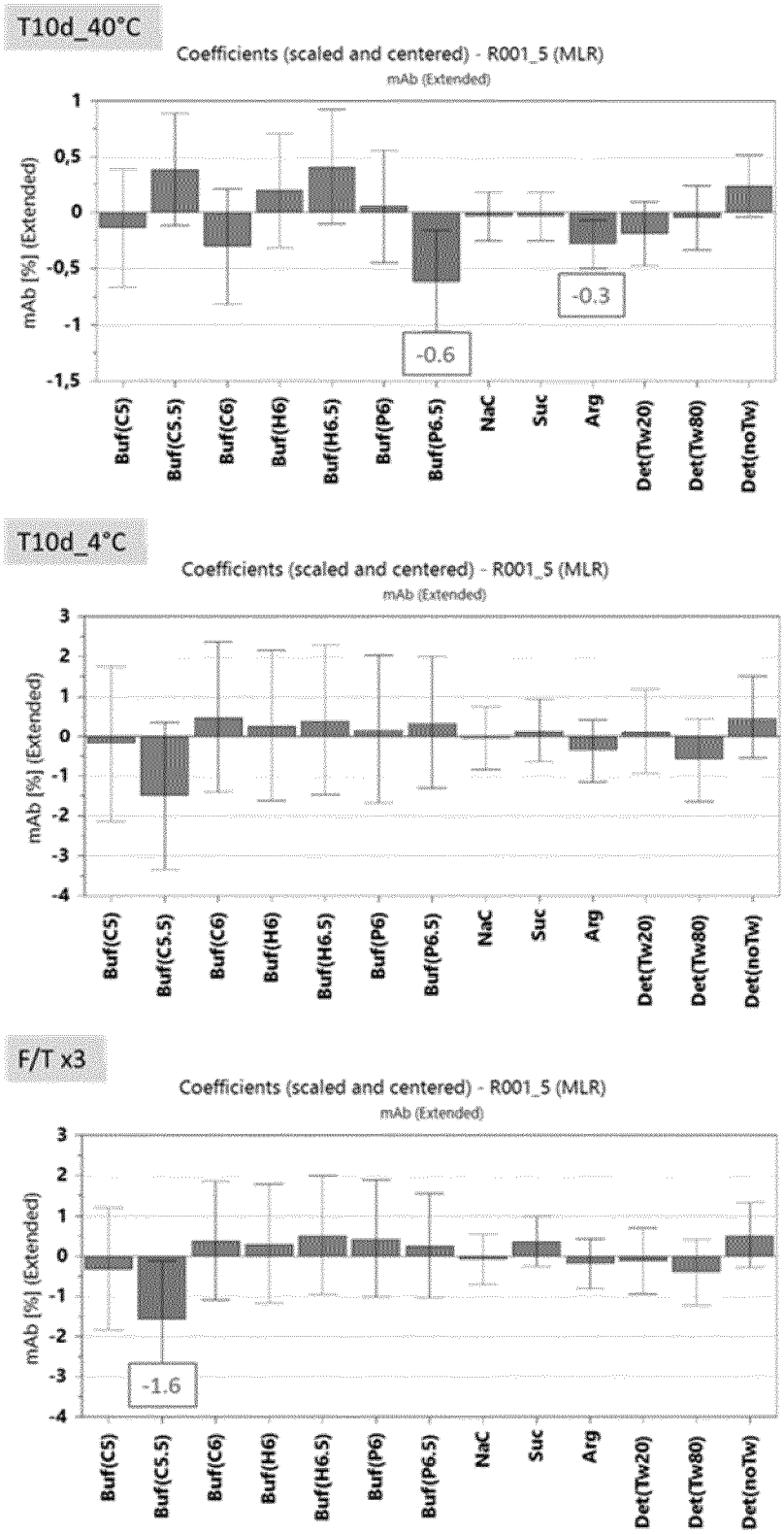


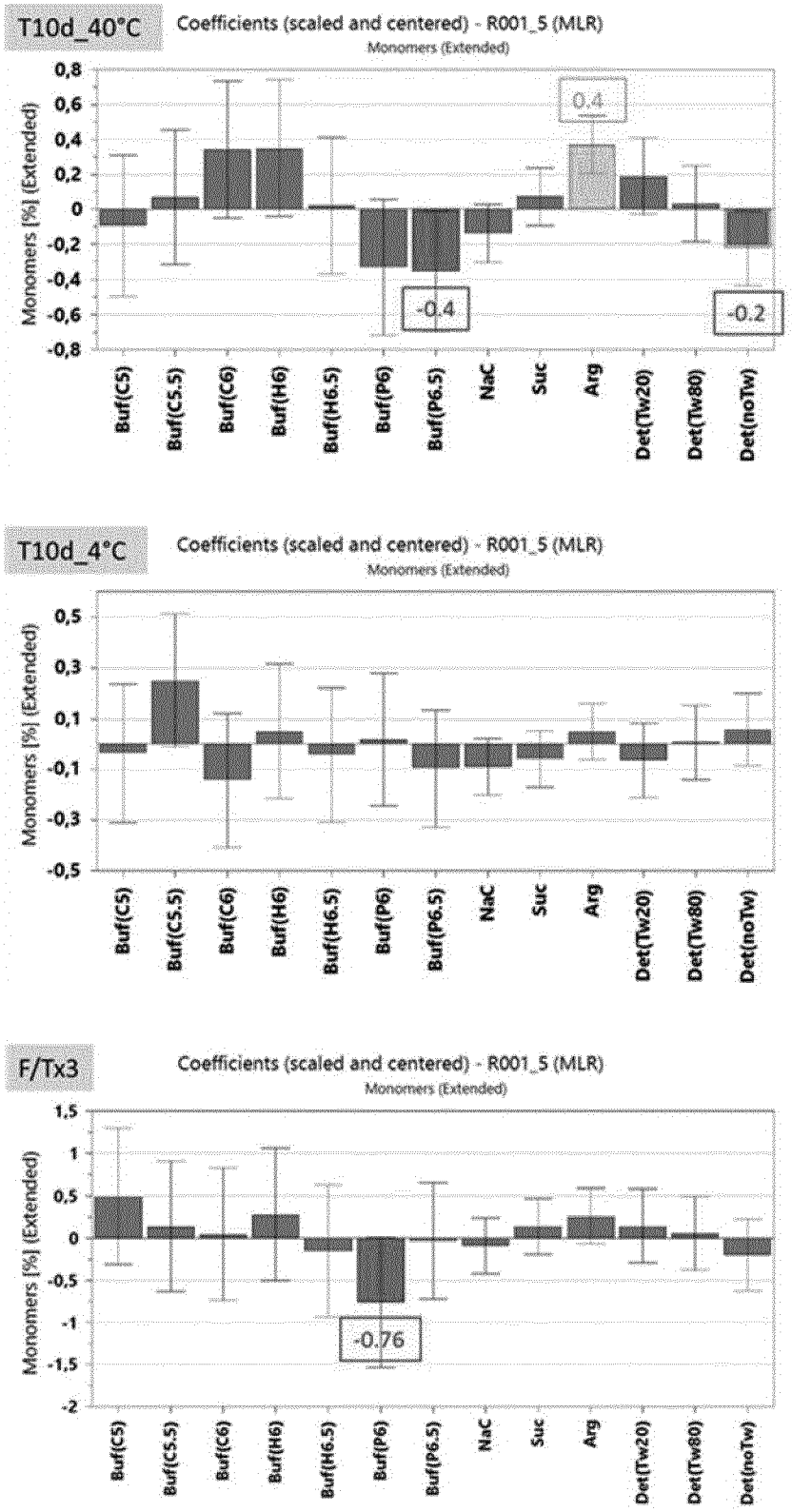
Fig. 12

A)

		T0	T10d_40°C	T10d_4°C	F/Tx3
1	C5 Nac Suc Arg Tw20	98,4	98,4	99,0	99,1
2	C5.5 Arg Tw20	98,5	98,8	98,9	98,6
3	C6 Nac Tw20	98,7	98,3	98,1	98,4
4	H6 Nac Tw20	98,7	98,6	98,6	98,8
5	H6.5 Suc Tw20	98,5	98,2	98,2	98,4
6	P6 Suc Arg Tw20	98,7	99,1	98,7	98,7
7	P6.5 Nac Arg Tw20	98,6	98,5	98,4	98,2
8	C5 Suc Tw80	98,8	98,2	98,6	98,8
9	C5.5 Nac Arg Tw80	98,9	98,6	98,8	98,6
10	C6 Suc Arg Tw80	98,7	99,2	98,3	98,6
11a	H6 Tw80	98,5	98,4	98,5	98,2
11b	H6 Tw80	98,3	98,8	98,2	98,2
11c	H6 Tw80	98,5	97,8	98,4	98,2
12	H6.5 Nac Arg Tw80	98,6	98,5	98,5	98,3
13	P6 Nac Arg Tw80	98,5	98,4	98,4	98,7
14	P6.5 Nac Suc Tw80	98,5	97,6	98,4	98,4
15	C5	98,8	98,0	98,7	98,9
16	C5.5 Nac Suc	98,7	98,0	98,5	98,6
17	C6 Arg	98,4	99,0	98,8	98,7
18	H6 Nac Suc Arg	98,3	98,6	98,5	98,7
19	H6.5 Nac Suc Arg	98,6	98,7	98,5	98,5
20	P6 Nac	98,5	96,7	98,4	95,7
21	P6.5 Arg	98,5	98,2	98,5	98,6
22	P6.5 Nac Suc Arg	98,2	97,6	98,3	98,3
Samples 11	Mean	98.4	98.3	98.4	98.2
	SD	0.15	0.5	0.16	0

Fig. 12 (continued)

B)



INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/061555

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. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.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/EP2023/061555

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61P35/00 G01N33/574
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61P A61K G01N

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)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/097536 A2 (JANSSEN PHARMACEUTICA NV [BE]) 2 July 2015 (2015-07-02) claims 1-102; figure 37; examples 4,6-9,12,15,16,20-22	1-22
X	US 10 577 424 B1 (HU WENQI [US] ET AL) 3 March 2020 (2020-03-03) claims 1-19; examples 2-4,7-8,11,13	1-22
X	WO 2020/016459 A1 (PF MEDICAMENT [FR]) 23 January 2020 (2020-01-23) page 79 lines 2-3,pages 92-107, pages 123-124, page 69; claim 10; figure 13; table 5; sequences 6,32,16,20,24,26,30,36	1-22

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 July 2023	09/08/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mattugini, Nicola

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2022/229469 A1 (PF MEDICAMENT [FR]) 3 November 2022 (2022-11-03) page 74 - page 75; claims 1,7,11,12-18; sequences 21,22	1,16-22
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