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(73) Patenthaver: **Bristol-Myers Squibb Company, Route 206 and Province Line Road, Princeton, NJ 08543, USA**

(72) Opfinder: **LONBERG, Nils, c/o Bristol-Myers Squibb Company, 700 Bay Road, Redwood City, CA 94063, USA**
SRINIVASAN, Mohan, c/o Bristol-Myers Squibb Company, 700 Bay Road, Redwood City, CA 94063, USA

(74) Fuldmægtig i Danmark: **Marks & Clerk LLP, 44 rue de la Vallée, BP 1775, L-1017 Luxembourg, Luxembourg**

(54) Benævnelse: **OPTIMERING AF ANTISTOFFER, DER BINDER LYMFOCYTAKTIVERINGSGEN-3 (LAG-3), OG ANVENDELSER DERAF**

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DESCRIPTION

Description

Background of the Invention

[0001] Therapeutic antibodies are one of the fastest growing segments of the pharmaceutical industry. To maintain potency (i.e., activity) and minimize immunogenicity, antibodies and other protein drugs must be protected from physical and chemical degradation during manufacturing and storage. Indeed, one of the primary difficulties in developing antibody therapeutics is the potential immunogenic response when administered to a subject, which can lead to rapid clearance or even induce life-threatening side effects including anaphylactic shock. Various factors influence the immunogenicity of an antibody such as its physiochemical properties (e.g., purity, stability, or solubility), clinical factors (e.g., dose, route of administration, heterogeneity of the disease, or patient features), and concomitant treatment with other agents (Swann et al. (2008) Curr Opinion Immuol 20:493-499).

[0002] Immunogenicity of antibodies and/or loss of antibody activity is often due to deamidation. Deamidation is a chemical degradative process that spontaneously occurs in proteins (e.g., antibodies). Deamidation removes an amide functional group from an amino acid residue, such as asparagine and glutamine, thus damaging its amide-containing side chains. This, in turn, causes structural and biological alterations throughout the protein, thus creating heterogeneous forms of the antibody. Deamidation is one of the most common post-translational modifications that occur in recombinantly produced therapeutic antibodies.

[0003] For example, heterogeneity in the heavy chain of monoclonal antibody h1B4 (a humanized anti-CD18 antibody) due to deamidation during cell culture was reported by Tsai et al. (Pharm Res 10(11): 1580 (1993)). In addition, reduction/loss of biological activity due to deamidation has been a recognized problem. For example, Kroon et al. characterized several deamidation sites in therapeutic antibody OKT3, and reported that samples of OKT3 production lots (aged 14 months to 3 years) had fallen below 75% activity (Pharm Res 9(11):1386 (1992), page 1389, second column). In addition, samples of OKT3 showing large amounts of the oxidized peptides in their maps had significantly reduced activity in the antigen binding potency assay (page 1390, first column). The authors concluded that specific sites of chemical modification that occur upon storage of OKT3 were identified by peptide mapping and correlated with observed changes in chemical analyses and biological assays of the antibody (page 1392, first column). Loss of biological activity also has been reported for a variety of other deamidated therapeutic proteins, including recombinant human DNase (Cacia et al. (1993) J. Chromatogr. 634:229-239) and recombinant soluble CD4 (Teshima et al. (1991) Biochemistry 30:3916-3922). WO 2010/019570 A2 describes a combination therapy in which

an anti- LAG-3 antibody is co-administered with at least one additional immunostimulatory antibody, such as an anti-PD-1 antibody, and a pharmaceutically acceptable carrier.

[0004] Overall, deamidation poses a significant and unpredictable problem to the pharmaceutical industry. Efforts associated with monitoring the variability caused by deamidation within antibody therapeutics, in particular, as well as FDA concerns associated with this variability, increase costs and delay clinical trials. Moreover, modifications to address this issue, including shifting conditions (e.g., temperature, pH, and cell type) associated with recombinant production and/or alteration of amino acids which are susceptible to deamidation (e.g., site-directed mutagenesis) can negatively impact stability and activity, especially when changes are made within the complementarity determining regions (CDRs) of the antibody. Accordingly, the need exists for more stable versions of therapeutic antibodies.

Summary

[0005] The present invention is defined by the claims. It provides a single composition comprising: (a) a monoclonal antibody, or an antigen-binding portion thereof, that binds human lymphocyte activation gene-3 (LAG-3), (b) an anti-PD-1 antibody, or an antigen-binding portion thereof, and (c) a pharmaceutically acceptable carrier, wherein the monoclonal antibody or antigen-binding portion thereof that binds human LAG-3 comprises heavy chain CDR1, CDR2, and CDR3 regions comprising the amino acid sequences of SEQ ID NOs: 15, 16, and 17, respectively, and light chain CDR1, CDR2, and CDR3 regions comprising the amino acid sequences of SEQ ID NOs: 18, 19, and 20, respectively.

[0006] In particular, the invention may use isolated monoclonal antibodies (e.g., human monoclonal antibodies) that bind LAG-3 (e.g., human LAG-3). The antibodies that bind human LAG-3 have optimized physical stability compared to previously described anti-LAG-3 antibodies. In particular, the invention uses a modified form of antibody 25F7 (US 2011/0150892 A1) which exhibits significantly improved thermal and chemical stability compared to the unmodified antibody. Specifically, by altering the critical binding region of the heavy chain CDR2 domain of antibody 25F7, it was shown that the modified antibody exhibited significantly higher physical and thermal stability, reduced deamidation, higher thermal reversibility, and lower aggregation. At the same time, it was unexpectedly observed that the modified antibody retained the same high binding affinity to human LAG-3 and functional activity of the unmodified antibody, including the ability to inhibit binding of LAG-3 to major histocompatibility (MHC) Class II molecules and stimulate antigen-specific T cell responses. The combined substantial increase in stability and retention of binding / biological activity of the modified antibody was surprising, particularly in view of the criticality of CDRs regions to antibody function.

[0007] The compositions of the invention can be used for a variety of applications, including stimulation of antigen-specific T cell responses in tumor-bearing or virus-bearing subjects, for inhibiting growth of tumor cells in a subject, or for treating cancer in a subject.

[0008] Accordingly, in one aspect, the invention pertains to a composition comprising a monoclonal antibody (e.g., a human antibody), or an antigen-binding portion thereof, having a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 12. In another embodiment, the antibody further includes a light chain variable region comprising the amino acid sequence of SEQ ID NO: 14.

[0009] In a preferred embodiment the antibody that binds human LAG-3 exhibits increased physical properties (i.e., thermal and chemical stability) compared to antibody 25F7, while still retaining at least the same binding affinity for human LAG-3 as 25F7. For example, the antibody exhibits decreased sequence variability in the heavy chain CDR2 region due to deamidation, compared to antibody 25F7, e.g., approximately 2.5% or less modification of the amino acid sequence after 12 weeks at 4C° (i.e., under "real-time" stability studies as described herein) and/or approximately 12.0% or less modification of the amino acid sequence after 12 weeks at 40C° (i.e., under accelerated stress conditions, as described herein), while still retaining a binding affinity for human LAG-3 of about at least K_D of 1×10^{-7} M or less (more preferably, a K_D of 1×10^{-8} M or less, a K_D of 5×10^{-9} M or less, or a K_D of 1×10^{-9} M or less). In another embodiment, the antibody exhibits thermal reversibility of at least about 40% in PBS at pH8.0. In another embodiment, the antibody that binds human LAG-3 possesses a higher melting temperature (indicating greater overall stability *in vivo*), compared to the unmodified antibody (Krishnamurthy R and Manning MC (2002) Curr Pharm Biotechnol 3:361-71). In one embodiment, the antibody exhibits a T_{M1} (the temperature of initial unfolding) of greater than 60°C, e.g., greater than 65°C, or greater than 70°C. The melting point of an antibody can be measured using differential scanning calorimetry (Chen et al (2003) Pharm Res 20:1952-60; Ghirlando et al (1999) Immunol Lett 68:47-52) or circular dichroism (Murray et al. (2002) J. Chromatogr Sci 40:343-9).

[0010] In another embodiment, the antibody that binds human LAG-3 is characterized by its resistance to rapid degradation. Degradation of an antibody can be measured using capillary electrophoresis (CE) and MALDI-MS (Alexander AJ and Hughes DE (1995) Anal Chem 67:3626-32).

[0011] In another embodiment, the antibody that binds human LAG-3 exhibits minimal aggregation effects, e.g., aggregation of 25% or less, such as 20% or less, 15% or less, 10% or less, 5% or less, or 4% or less. Aggregation can lead to the triggering of an unwanted immune response and/or altered or unfavorable pharmacokinetic properties. Aggregation can be measured by several techniques, including size-exclusion column (SEC), high performance liquid chromatography (HPLC), and light scattering.

[0012] In another embodiment, the antibody that binds human LAG-3 further exhibits at least one of the following properties:

1. (a) binding to monkey LAG-3;

2. (b) lack of binding to mouse LAG-3;
3. (c) inhibition of binding of LAG-3 to major histocompatibility (MHC) class II molecules; and
4. (d) stimulation of immune responses, particularly antigen-specific T cell responses.

Preferably, the antibody exhibits at least two of properties (a), (b), (c) and (d). More preferably, the antibody exhibits at least three of properties (a), (b), (c) and (d). Even more preferably, the antibody exhibits all four of properties (a), (b), (c) and (d).

[0013] In another embodiment, the antibody that binds human LAG-3 stimulates an antigen-specific T cell response, such as interleukin-2 (IL-2) production in an antigen-specific T cell response. In other embodiments, the antibody stimulates an immune response, such as an anti-tumor response (*e.g.,* inhibition of tumor growth in an *in vivo* tumor graft model) or an autoimmune response (*e.g.,* development of diabetes in NOD mice).

[0014] In another embodiment, the antibody that binds human LAG-3 binds an epitope of human LAG-3 comprising the amino acid sequence PGHPLAPG (SEQ ID NO: 21). In another embodiment, the antibody binds an epitope of human LAG-3 comprising the amino acid sequence HPAAPSSW (SEQ ID NO: 22) or PAAPSSWG (SEQ ID NO: 23).

[0015] In other embodiments, the antibody that binds human LAG-3 stains pituitary tissue by immunohistochemistry, or does not stain pituitary tissue by immunohistochemistry.

[0016] Antibodies of the invention can be full-length antibodies, for example, of an IgG1, IgG2 or IgG4 isotype, optionally with a serine to proline mutation in the heavy chain constant region hinge region (at a position corresponding to position 241 as described in Angal et al. (1993) Mol. Immunol. 30:105-108), such that inter-heavy chain disulfide bridge heterogeneity is reduced or abolished. In one aspect, the constant region isotype is IgG4 with a mutation at amino acid residues 228, e.g., S228P. Alternatively, the antibodies can be antibody fragments, such as Fab, Fab' or Fab'2 fragments, or single chain antibodies.

[0017] In another aspect of the invention, the antibody (or antigen-binding portion thereof) is part of an immunoconjugate which includes a therapeutic agent, *e.g.,* a cytotoxin or a radioactive isotope, linked to the antibody. In another aspect, the antibody is part of a bispecific molecule which includes a second functional moiety (e.g., a second antibody) having a different binding specificity than said antibody, or antigen binding portion thereof.

[0018] Nucleic acid molecules encoding the antibodies, or antigen-binding portions (e.g., variable regions and/or CDRs) thereof, of the invention also are described, as well as expression vectors comprising such nucleic acids and host cells comprising such expression vectors. Methods for preparing anti-LAG-3 antibodies using the host cells comprising such expression vectors also are described, and can include the steps of (i) expressing the antibody in the host cell and (ii) isolating the antibody from the host cell.

[0019] In another aspect, the invention provides the compositions of the invention for use in methods of stimulating immune responses using of the invention. In one embodiment, the method involves stimulating an antigen-specific T cell response by contacting T cells with a composition of the invention, such that an antigen-specific T cell response is stimulated. In a preferred embodiment, interleukin-2 production by the antigen-specific T cell is stimulated. In another embodiment, the subject is a tumor-bearing subject and an immune response against the tumor is stimulated. In another embodiment, the subject is a virus-bearing subject and an immune response against the virus is stimulated.

[0020] In yet another embodiment, the invention provides the compositions of the invention for use in a method for inhibiting growth of tumor cells in a subject comprising administering to the subject a composition of the invention, such that growth of the tumor is inhibited in the subject. In still another embodiment, the invention provides the compositions of the invention for use in a method for treating viral infection in a subject comprising administering to the subject a composition of the invention such that the viral infection is treated in the subject.

[0021] Other features and advantages of the instant disclosure will be apparent from the following detailed description and examples, which should not be construed as limiting.

Brief Description of the Drawings

[0022]

Figure 1A shows the nucleotide sequence (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO: 2) of the heavy chain variable region of the 25F7 human monoclonal antibody. The CDR1 (SEQ ID NO: 5), CDR2 (SEQ ID NO: 6) and CDR3 (SEQ ID NO: 7) regions are delineated and the V, D and J germline derivations are indicated. The CDR regions are delineated using the Kabat system (Kabat et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242).

Figure 1B shows the nucleotide sequence (SEQ ID NO: 3) and amino acid sequence (SEQ ID NO: 4) of the kappa light chain variable region of the 25F7 human monoclonal antibody. The CDR1 (SEQ ID NO: 8), CDR2 (SEQ ID NO: 9) and CDR3 (SEQ ID NO: 10) regions are delineated and the V and J germline derivations are indicated. The full-length heavy and light chain amino acid sequences of antibody 25F7 are shown in SEQ ID NOs: 32 and 34, respectively.

Figure 2A shows the amino acid sequence (SEQ ID NO: 12) of the heavy chain variable region of the LAG3.5 monoclonal antibody. The CDR1 (SEQ ID NO: 15), CDR2 (SEQ ID NO: 16) and CDR3 (SEQ ID NO: 17) regions are delineated. The full-length heavy and light chain amino acid sequences of antibody LAG3.5 are shown in SEQ ID NOs: 35 and 37, respectively.

Figure 2B shows the nucleotide sequence (SEQ ID NO: 13) and amino acid sequence (SEQ ID NO: 14) of the kappa light chain variable region of the LAG3.5 monoclonal antibody. The CDR1

(SEQ ID NO: 18), CDR2 (SEQ ID NO: 19) and CDR3 (SEQ ID NO: 20) regions are delineated.

Figure 3 shows the amino acid sequences of the CDR2 heavy chain variable region sequences of the LAG-3 variants LAG3.5 (SEQ ID NO: 42), LAG3.6 (SEQ ID NO: 43), LAG3.7 (SEQ ID NO: 44), and LAG3.8 (SEQ ID NO: 45), compared to the amino acid sequence of the CDR2 heavy chain variable region sequence of antibody 25F7 (LAG3.1) (SEQ ID NO: 41) and corresponding human germline sequence (SEQ ID NO: 27). The CDR2 heavy chain variable region of LAG3.5 differs from the CDR2 heavy chain variable region of 25F7 by arginine (R) at position 54 (versus asparagine (N)) and serine (S) at position 56 (versus asparagine (N)). The remaining CDRs of LAG3.5 and 25F7 are identical. Figure 3 also discloses SEQ ID NO: 40.

Figures 4A and 4B are graphs showing the binding activity (EC_{50} and affinity, respectively) of antibodies LAG3.1 (25F7), LAG3.2, LAG3.5, LAG3.6, LAG3.7, and LAG3.8 to activated human CD4+ T cells. Figure 4B discloses SEQ ID NOS 41, 42, 45, 44, and 43, respectively, in order of appearance.

Figures 5A, B, C, D, and E are graphs showing thermal melting curves (i.e., thermal stability) of antibodies LAG3.1 (25F7), LAG3.5, LAG3.6, LAG3.7, and LAG3.8, respectively.

Figures 6A, B, C, D, and E are graphs showing thermal reversibility curves (i.e., thermal stability) of antibodies LAG3.1 (25F7), LAG3.5, LAG3.6, LAG3.7, and LAG3.8, respectively.

Figure 7 is a graph, showing the binding activity of antibodies LAG3.1 (25F7) and LAG3.5 to activated human CD4+ T cells and antigen binding (Biacore).

Figure 8 shows the results of peptide mapping using mass-spectrometry (chemical modifications / molecular stability) for antibodies LAG3.1 (25F7) and LAG3.5 reflecting deamidation and isomerization after incubating for 5 days under accelerated stress conditions as described herein. Figure 8 discloses SEQ ID NOS 46-52, respectively, in order of appearance.

Figure 9 is a graph comparing the hydrophilicity profiles of antibodies LAG3.1 (25F7) and LAG3.5.

Figures 10 A, B, C, and D are graphs comparing the affinity and physical stability (i.e., thermal and chemical stability) of antibodies LAG3.1 and LAG3.5 at 4C° and 40C°, i.e., both accelerated stress conditions and "real-time" stability studies, as described herein.

Figures 11 A and B are graphs comparing the percent modification of the amino acid sequences of antibodies LAG3.1 and LAG3.5 at 4C° and 40C°.

Detailed Description of the Invention

[0023] In order that the present disclosure may be more readily understood, certain terms are

first defined. Additional definitions are set forth throughout the detailed description.

[0024] The terms "25F7," "antibody 25F7," "antibody LAG3.1," and "LAG3.1" refer to the anti-human LAG-3 antibody described in US2011/0150892 A1. The nucleotide sequence (SEQ ID NO: 1) encoding the heavy chain variable region of 25F7 (LAG3.1) and the corresponding amino acid sequence (SEQ ID NO: 2) is shown in Figure 1A (with CDR sequences designated as SEQ ID NOs: 4, 5, and 7, respectively). The nucleotide sequence (SEQ ID NO: 3) encoding the light chain variable region of 25F7 (LAG3.1) and the corresponding amino acid sequence (SEQ ID NO: 4) is shown in Figure 1B (with CDR sequences designated as SEQ ID NOs: 8, 9, and 10, respectively).

[0025] The term "LAG-3" refers to Lymphocyte Activation Gene-3. The term "LAG-3" includes variants, isoforms, homologs, orthologs and paralogs. For example, antibodies specific for a human LAG-3 protein may, in certain cases, cross-react with a LAG-3 protein from a species other than human. In other embodiments, the antibodies specific for a human LAG-3 protein may be completely specific for the human LAG-3 protein and may not exhibit species or other types of cross-reactivity, or may cross-react with LAG-3 from certain other species but not all other species (*e.g.,* cross-react with monkey LAG-3 but not mouse LAG-3). The term "human LAG-3" refers to human sequence LAG-3, such as the complete amino acid sequence of human LAG-3 having Genbank Accession No. NP_002277 (SEQ ID NO: 29). The term "mouse LAG-3" refers to mouse sequence LAG-3, such as the complete amino acid sequence of mouse LAG-3 having Genbank Accession No. NP_032505. LAG-3 is also known in the art as, for example, CD223. The human LAG-3 sequence may differ from human LAG-3 of Genbank Accession No. NP_002277 by having, *e.g.,* conserved mutations or mutations in non-conserved regions and the LAG-3 has substantially the same biological function as the human LAG-3 of Genbank Accession No. NP_002277. For example, a biological function of human LAG-3 is having an epitope in the extracellular domain of LAG-3 that is specifically bound by an antibody of the instant disclosure or a biological function of human LAG-3 is binding to MHC Class II molecules.

[0026] The term "monkey LAG-3" is intended to encompass LAG-3 proteins expressed by Old World and New World monkeys, including but not limited to cynomolgus monkey LAG-3 and rhesus monkey LAG-3. A representative amino acid sequence for monkey LAG-3 is the rhesus monkey LAG-3 amino acid sequence which is also deposited as Genbank Accession No. XM _001108923. Another representative amino acid sequence for monkey LAG-3 is the alternative rhesus monkey sequence of clone pa23-5 as described in US 2011/0150892 A1. This alternative rhesus sequence exhibits a single amino acid difference, at position 419, as compared to the Genbank-deposited sequence.

[0027] A particular human LAG-3 sequence will generally be at least 90% identical in amino acid sequence to human LAG-3 of Genbank Accession No. NP_002277 and contains amino acid residues that identify the amino acid sequence as being human when compared to LAG-3 amino acid sequences of other species (*e.g.,* murine). In certain cases, a human LAG-3 can be at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to

LAG-3 of Genbank Accession No. NP_002277. In certain embodiments, a human LAG-3 sequence will display no more than 10 amino acid differences from the LAG-3 sequence of Genbank Accession No. NP_002277. In certain embodiments, the human LAG-3 can display no more than 5, or even no more than 4, 3, 2, or 1 amino acid difference from the LAG-3 sequence of Genbank Accession No. NP_002277. Percent identity can be determined as described herein.

[0028] The term "immune response" refers to the action of, for example, lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

[0029] An "antigen-specific T cell response" refers to responses by a T cell that result from stimulation of the T cell with the antigen for which the T cell is specific. Non-limiting examples of responses by a T cell upon antigen-specific stimulation include proliferation and cytokine production (*e.g.,* IL-2 production).

[0030] The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. Whole antibodies are glycoproteins comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_H1, C_H2 and C_H3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, 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 can 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 (Clq) of the classical complement system.

[0031] The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.,* a LAG-3 protein). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_H1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide

bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_H and C_H1 domains; (v) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (vi) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a V_H domain; (vii) an isolated complementarity determining region (CDR); and (viii) a nanobody, a heavy chain variable region containing a single variable domain and two constant domains. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); *see e.g.,* Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

[0032] An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.,* an isolated antibody that specifically binds a LAG-3 protein is substantially free of antibodies that specifically bind antigens other than LAG-3 proteins). An isolated antibody that specifically binds a human LAG-3 protein may, however, have cross-reactivity to other antigens, such as LAG-3 proteins from other species. Moreover, an isolated antibody can be substantially free of other cellular material and/or chemicals.

[0033] The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

[0034] The term "human antibody", as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies of the invention can include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.,* mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0035] The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity, which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.,* a transgenic mouse, having a genome comprising a human

heavy chain transgene and a light chain transgene fused to an immortalized cell.

[0036] The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.,* a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.,* from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo.*

[0037] The term "isotype" refers to the antibody class (*e.g.,* IgM or IgGI) that is encoded by the heavy chain constant region genes.

[0038] The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

[0039] The term "human antibody derivatives" refers to any modified form of the human antibody, *e.g.,* a conjugate of the antibody and another agent or antibody.

[0040] The term "humanized antibody" is intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications can be made within the human framework sequences.

[0041] The term "chimeric antibody" is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

[0042] As used herein, an antibody that "specifically binds human LAG-3" is intended to refer to an antibody that binds to human LAG-3 protein (and possibly a LAG-3 protein from one or more non-human species) but does not substantially bind to non-LAG-3 proteins. Preferably, the antibody binds to a human LAG-3 protein with "high affinity", namely with a K_D of 1×10^{-7}

M or less, more preferably 1×10^{-8} M or less, more preferably 5×10^{-9} M or less, more preferably 1×10^{-9} M or less.

[0043] The term "does not substantially bind" to a protein or cells, as used herein, means does not bind or does not bind with a high affinity to the protein or cells, *i.e.* binds to the protein or cells with a K_D of 1×10^{-6} M or more, more preferably 1×10^{-5} M or more, more preferably 1×10^{-4} M or more, more preferably 1×10^{-3} M or more, even more preferably 1×10^{-2} M or more.

[0044] The term "K_{assoc}" or "K_a", as used herein, is intended to refer to the association rate of a particular antibody-antigen interaction, whereas the term "K_{dis}" or "K_d," as used herein, is intended to refer to the dissociation rate of a particular antibody-antigen interaction. The term "K_D," as used herein, is intended to refer to the dissociation constant, which is obtained from the ratio of Kato K_a (*i.e.,* K_d/K_a) and is expressed as a molar concentration (M). K_D values for antibodies can be determined using methods well established in the art. A preferred method for determining the K_D of an antibody is by using surface plasmon resonance, preferably using a biosensor system such as a Biacore® system.

[0045] The term "high affinity" for an IgG antibody refers to an antibody having a K_D of 1×10^{-7} M or less, more preferably 5×10^{-8} M or less, even more preferably 1×10^{-8} M or less, even more preferably 5×10^{-9} M or less and even more preferably 1×10^{-9} M or less for a target antigen. However, "high affinity" binding can vary for other antibody isotypes. For example, "high affinity" binding for an IgM isotype refers to an antibody having a K_D of 10^{-6} M or less, more preferably 10^{-7} M or less, even more preferably 10^{-8} M or less.

[0046] The term "deamidation" refers to a chemical degradative process that spontaneously occurs in proteins (e.g., antibodies). Deamidation removes an amide functional group from an amino acid residue, such as asparagine and glutamine, thus damaging its amide-containing side chains. Specifically, the side chain of an asparagine attacks the adjacent peptide group, forming a symmetric succinimide intermediate. The symmetry of the intermediate results in two hydrolysis products, either aspartate or isoaspartate. A similar reaction can also occur in aspartate side chains, yielding a partial conversion to isoaspartate. In the case of glutamine, the rate of deamidation is generally ten fold less than asparagine, however, the mechanism is essentially the same, requiring only water molecules to proceed.

[0047] The term "subject" includes any human or nonhuman animal. The term "nonhuman animal" includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, amphibians, and reptiles, although mammals are preferred, such as non-human primates, sheep, dogs, cats, cows and horses.

[0048] Various aspects of the invention are described in further detail in the following subsections.

Anti-LAG-3 Antibodies Having Increased Stability and Advantageous Functional Properties

[0049] Antibodies which bind human LAG-3 for use in the invention specifically bind to human LAG-3 and have optimized stability compared to previously described anti-LAG-3 antibodies, particularly compared to antibody 25F7 (LAG3.1). This optimization includes reduced deamidation (e.g., increased chemical stability) and increased thermal refolding (e.g., increased physical stability), while still retaining high affinity binding to human LAG-3.

[0050] Methods for identifying deamidation sites are known in the art (see, e.g., ion exchange, reversed phase, and hydrophobic interaction chromatography, and peptide mapping of proteolytic digests (LC-MS)). Suitable assays for measuring physical stability include, e.g., analysis of melting points and/or refolding of antibody structure following denaturation (e.g., percent reversibility as described, e.g., in Example 3, Section 3).

[0051] Binding to human LAG-3 can be assessed using one or more techniques also well established in the art. For example, an antibody can be tested by a flow cytometry assay in which the antibody is reacted with a cell line that expresses human LAG-3, such as CHO cells that have been transfected to express LAG-3 (*e.g.,* human LAG-3, or monkey LAG-3 (*e.g.,* rhesus or cynomolgus monkey) or mouse LAG-3) on their cell surface. Other suitable cells for use in flow cytometry assays include anti-CD3-stimulated $CD4^+$ activated T cells, which express native LAG-3. Additionally or alternatively, binding of the antibody, including the binding kinetics (*e.g.,* K_D value), can be tested in BIAcore assays. Still other suitable binding assays include ELISA assays, for example, using a recombinant LAG-3 protein.

[0052] Antibodies which bind human LAG-3 preferably bind to human LAG-3 protein with a K_D of 1×10^{-7} M or less, and more preferably 1×10^{-8} M or less, 5×10^{-9} M or less, or 1×10^{-9} M or less.

[0053] Typically, the antibody binds to LAG-3 in lymphoid tissues, such as tonsil, spleen or thymus, which can be detected by immunohistochemistry. In one embodiment, the antibody stains pituitary tissue (*e.g.,* are retained in the pituitary) as measured by immunohistochemistry. In another embodiment, the antibody does not stain pituitary tissue (*i.e.,* is not retained in the pituitary) as measured by immunohistochemistry.

[0054] Additional functional properties include cross-reactivity with LAG-3 from other species. For example, the antibody can bind to monkey LAG-3 (*e.g.,* cynomolgus monkey, rhesus monkey), but not substantially bind to LAG-3 from mouse LAG-3. Preferably, an antibody which binds human LAG-3binds to human LAG-3 with high affinity.

[0055] Other functional properties include the ability of the antibody to stimulate an immune

response, such as an antigen-specific T cell response. This can be tested, for example, by assessing the ability of the antibody to stimulate interleukin-2 (IL-2) production in an antigen-specific T cell response. In certain embodiments, the antibody binds to human LAG-3 and stimulates an antigen-specific T cell response. In other embodiments, the antibody binds to human LAG-3 but does not stimulate an antigen-specific T cell response. Other means for evaluating the capacity of the antibody to stimulate an immune response include testing its ability to inhibit tumor growth, such as in an *in vivo* tumor graft model *(see, e.g.,* Example 6) or the ability to stimulate an autoimmune response, such as the ability to promote the development of an autoimmune disease in an autoimmune model, e.g., the ability to promote the development of diabetes in the NOD mouse model.

[0056] Preferred antibodies for use in the invention are human monoclonal antibodies. Additionally or alternatively, the antibodies can be, for example, chimeric or humanized monoclonal antibodies.

Monoclonal Antibody LAG3.5

[0057] An antibody which binds human LAG-3 and is preferred for use in the invention is the human monoclonal antibody, LAG3.5, structurally and chemically characterized as described below and in the following Examples. The V_H amino acid sequence of LAG3.5 is shown in SEQ ID NO: 12 (Figure 2A). The V_L amino acid sequence of LAG3.5 is shown in SEQ ID NO: 14 (Figure 2B).

[0058] The V_H and V_L sequences (or CDR sequences) of other anti-LAG-3 antibodies which bind human LAG-3 can be "mixed and matched" with the V_H and V_L sequences (or CDR sequences) of antibody LAG3.5. Preferably, when V_H and V_L chains (or the CDRs within such chains) are mixed and matched, a V_H sequence from a particular V_H/V_L pairing is replaced with a structurally similar V_H sequence. Likewise, preferably a V_L sequence from a particular V_H/V_L pairing is replaced with a structurally similar V_L sequence.

[0059] Accordingly antibodies which bind human LAG-3may comprise:

1. (a) a heavy chain variable region comprising amino acid sequence SEQ ID NO: 12 (i.e., the V_H of LAG3.5); and
2. (b) a light chain variable region comprising amino acid sequence SEQ ID NO: 14 (i.e., the V_L of LAG3.5) or the V_L of another anti-LAG3 antibody (i.e., which differs from LAG3.5);

wherein the antibody specifically binds human LAG-3.

[0060] Further antibodies which bind human LAG-3 may comprise:

1. (a) the CDR1, CDR2, and CDR3 regions of the heavy chain variable region comprising amino acid sequence SEQ ID NO: 12 (i.e., the CDR sequences of LAG3.5, SEQ ID NOs: 15, 16, and 17, respectively); and
2. (b) the CDR1, CDR2, and CDR3 regions of the light chain variable region comprising amino acid sequence SEQ ID NO: 14 (i.e., the CDR sequences of LAG3.5, SEQ ID NOs: 18, 19, and 20, respectively) or the CDRs of another anti-LAG3 antibody (i.e., which differs from LAG3.5);

wherein the antibody specifically binds human LAG-3.

[0061] Yet further antibodies which bind human LAG-3 include the heavy chain variable CDR2 region of LAG3.5 combined with CDRs of other antibodies which bind human LAG-3, e.g., a CDR1 and/or CDR3 from the heavy chain variable region, and/or a CDR1, CDR2, and/or CDR3 from the light chain variable region of a different anti-LAG-3 antibody.

[0062] In addition, it is well known in the art that the CDR3 domain, independently from the CDR1 and/or CDR2 domain(s), alone can determine the binding specificity of an antibody for a cognate antigen and that multiple antibodies can predictably be generated having the same binding specificity based on a common CDR3 sequence. *See, e.g.,* Klimka et al., British J. of Cancer 83(2):252-260 (2000); Beiboer et al., J. Mol. Biol. 296:833-849 (2000); Rader et al., Proc. Natl. Acad. Sci. U.S.A. 95:8910-8915 (1998); Barbas et al., J. Am. Chem. Soc. 116:2161-2162 (1994); Barbas et al., Proc. Natl. Acad. Sci. U.S.A. 92:2529-2533 (1995); Ditzel et al., J. Immunol. 157:739-749 (1996); Berezov et al., BIAjournal 8: Scientific Review 8 (2001); Igarashi et al., J. Biochem (Tokyo) 117:452-7 (1995); Bourgeois et al., J. Virol 72:807-10 (1998); Levi et al., Proc. Natl. Acad. Sci. U.S.A. 90:4374-8 (1993); Polymenis and Stoller, J. Immunol. 152:5218-5329 (1994) and Xu and Davis, Immunity 13:37-45 (2000). *See* also, US Patents Nos. 6,951,646; 6,914,128; 6,090,382; 6,818,216; 6,156,313; 6,827,925; 5,833,943; 5,762,905 and 5,760,185.

[0063] Accordingly, antibodies which bind human LAG-3 include the CDR2 of the heavy chain variable region of LAG3.5 and at least the CDR3 of the heavy and/or light chain variable region of LAG3.5 (SEQ ID NOs: 17 and/or 20), or the CDR3 of the heavy and/or light chain variable region of another LAG-3 antibody, wherein the antibody is capable of specifically binding to human LAG-3. These antibodies preferably (a) compete for binding with; (b) retain the functional characteristics; (c) bind to the same epitope; and/or (d) have a similar binding affinity as LAG3.5. Yet further antibodies which bind human LAG-3 may include the CDR2 of the light chain variable region of LAG3.5 (SEQ ID NOs: 17 and/or 20), or the CDR2 of the light chain variable region of another LAG-3 antibody, wherein the antibody is capable of specifically binding to human LAG-3. Other antibodies which bind human LAG-3may include the CDR1 of the heavy and/or light chain variable region of LAG3.5 (SEQ ID NOs: 17 and/or 20), or the CDR1 of the heavy and/or light chain variable region of another LAG-3 antibody, wherein the antibody is capable of specifically binding to human LAG-3.

Conservative Modifications

[0064] Other antibodies which bind human LAG-3 may comprise a heavy and/or light chain variable region sequences of CDR1, CDR2 and CDR3 sequences which differ from those of LAG3.5 by one or more conservative modifications. It is preferred, however, that residues 54 and 56 of the V_H CDR2 remain as arginine and serine, respectively (i.e., are not mutated). It is understood in the art that certain conservative sequence modification can be made which do not remove antigen binding. *See, e.g.,* Brummell et al. (1993) Biochem 32:1180-8; de Wildt et al. (1997) Prot. Eng. 10:835-41; Komissarov et al. (1997) J. Biol. Chem. 272:26864-26870; Hall et al. (1992) J. Immunol. 149:1605-12; Kelley and O'Connell (1993) Biochem. 32:6862-35; Adib-Conquy et al. (1998) Int. Immunol. 10:341-6 and Beers et al. (2000) Clin. Can. Res. 6:2835-43. Accordingly, the antibody comprises a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences and/or a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein:

1. (a) the heavy chain variable region CDR1 sequence comprises SEQ ID NO: 15, and/or conservative modifications thereof, except at positions 54 and 56; and/or
2. (b) the heavy chain variable region CDR3 sequence comprises SEQ ID NO: 17, and conservative modifications thereof; and/or
3. (c) the light chain variable region CDR1, and/or CDR2, and/or CDR3 sequences comprise SEQ ID NO: 18, and/or, SEQ ID NO: 19, and/or SEQ ID NO: 20, and/or conservative modifications thereof; and
4. (d) the antibody specifically binds human LAG-3.

[0065] Additionally or alternatively, the antibody can possess one or more of the following functional properties described above, such as high affinity binding to human LAG-3, binding to monkey LAG-3, lack of binding to mouse LAG-3, the ability to inhibit binding of LAG-3 to MHC Class II molecules and/or the ability to stimulate antigen-specific T cell responses.

[0066] The antibody described herein can be, for example, a human, humanized or chimeric antibody

[0067] As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.,* lysine, arginine, histidine), acidic side chains (*e.g.,* aspartic acid, glutamic acid), uncharged polar side chains *(e.g.,* glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.,*

alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.,* threonine, valine, isoleucine) and aromatic side chains (*e.g.,* tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of an antibody can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for retained function (*i.e.,* the functions set forth above) using the functional assays described herein.

Engineered and Modified Antibodies

[0068] Antibodies can be prepared using an antibody having one or more of the V_H and/or V_L sequences of LAG3.5 as starting material to engineer a modified antibody. An antibody can be engineered by modifying one or more residues within one or both variable regions (*i.e.,* V_H and/or V_L), for example within one or more CDR regions and/or within one or more framework regions. Additionally or alternatively, an antibody can be engineered by modifying residues within the constant region(s), for example to alter the effector function(s) of the antibody.

[0069] CDR grafting can be used to engineer variable regions of antibodies. Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (*see, e.g.,* Riechmann et al. (1998) Nature 332:323-327; Jones et al. (1986) Nature 321:522-525; Queen et al. (1989) Proc. Natl. Acad. See. U.S.A. 86:10029-10033; U.S. Pat. Nos. 5,225,539; 5,530,101; 5,585,089; 5,693,762 and 6,180,370).

[0070] Accordingly, another embodiment of the invention makes use of an isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences comprising SEQ ID NOs: 15, 16, 17, respectively, and a light chain variable region comprising CDR1, CDR2, and CDR3 sequences comprising SEQ ID NOs: 18, 19, 20, respectively (i.e., the CDRs of LAG3.5). While these antibodies contain the V_H and V_L CDR sequences of monoclonal antibody LAG3.5, they can contain differing framework sequences.

[0071] Such framework sequences can be obtained from public DNA databases or published references that include germline antibody gene sequences. For example, germline DNA sequences for human heavy and light chain variable region genes can be found in the "VBase" human germline sequence database (available on the Internet at www.mrc-cpe.cam.ac.uk/vbase), as well as in Kabat *et al.* (1991), cited *supra;* Tomlinson et al. (1992)

"The Repertoire of Human Germline VH Sequences Reveals about Fifty Groups of VH Segments with Different Hypervariable Loops" J. Mol. Biol. 227:776-798; and Cox et al. (1994) "A Directory of Human Germ-line VH Segments Reveals a Strong Bias in their Usage" Eur. J. Immunol. 24:827-836. As another example, the germline DNA sequences for human heavy and light chain variable region genes can be found in the Genbank database. For example, the following heavy chain germline sequences found in the HCo7 HuMAb mouse are available in the accompanying Genbank Accession Nos.: 1-69 (NG_0010109, NT_024637 & BC070333), 3-33 (NG_0010109 & NT_024637) and 3-7 (NG_0010109 & NT_024637). As another example, the following heavy chain germline sequences found in the HCo12 HuMAb mouse are available in the accompanying Genbank Accession Nos.: 1-69 (NG_0010109, NT_024637 & BC070333), 5-51 (NG_0010109 & NT_024637), 4-34 (NG_0010109 & NT_024637), 3-30.3 (CAJ556644) & 3-23 (AJ406678).

[0072] Antibody protein sequences are compared against a compiled protein sequence database using one of the sequence similarity searching methods called the Gapped BLAST (Altschul *et al.* (1997), *supra*), which is well known to those skilled in the art.

[0073] Preferred framework sequences for use in the antibodies are those that are structurally similar to the framework sequences used by selected antibodies, e.g., similar to the V_H 4-34 framework sequences and/or the V_K L6 framework sequences used by preferred monoclonal antibodies. The V_H CDR1, CDR2, and CDR3 sequences, and the V_K CDR1, CDR2, and CDR3 sequences, can be grafted onto framework regions that have the identical sequence as that found in the germline immunoglobulin gene from which the framework sequence derive, or the CDR sequences can be grafted onto framework regions that contain one or more mutations as compared to the germline sequences. For example, it has been found that in certain instances it is beneficial to mutate residues within the framework regions to maintain or enhance the antigen binding ability of the antibody (*see e.g.,* U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370).

[0074] Another type of variable region modification is to mutate amino acid residues within the V_H and/or V_L CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties (*e.g.,* affinity) of the antibody of interest. Site-directed mutagenesis or PCR-mediated mutagenesis can be performed to introduce the mutation(s) and the effect on antibody binding, or other functional property of interest, can be evaluated in *in vitro* or *in vivo* assays as described herein and provided in the Examples. Preferably conservative modifications (as discussed above) are introduced. The mutations can be amino acid substitutions, additions or deletions, but are preferably substitutions. Moreover, typically no more than one, two, three, four or five residues within a CDR region are altered.

[0075] Accordingly, isolated anti-LAG-3 monoclonal antibodies, or antigen binding portions thereof, may comprise a heavy chain variable region comprising: (a) a V_H CDR1 region comprising SEQ ID NO: 15, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NO: 15; (b) a V_H CDR2

region comprising SEQ ID NO: 16, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NO: 16 (preferably wherein positions 54 and 56 are the same as in SEQ ID NO: 16); (c) a V_H CDR3 region comprising SEQ ID NO: 17, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NO: 17; (d) a V_L CDR1 region comprising SEQ ID NO: 18, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NO: 18; (e) a V_L CDR2 region comprising SEQ ID NO: 19, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NO: 19; and (f) a V_L CDR3 region comprising SEQ ID NO: 20, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NO: 20.

[0076] Engineered antibodies include those in which modifications have been made to framework residues within V_H and/or V_L, *e.g.* to improve the properties of the antibody. Typically such framework modifications are made to decrease the immunogenicity of the antibody. For example, one approach is to "backmutate" one or more framework residues to the corresponding germline sequence. More specifically, an antibody that has undergone somatic mutation can contain framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody framework sequences to the germline sequences from which the antibody is derived.

[0077] Another type of framework modification involves mutating one or more residues within the framework region, to remove T cell epitopes to thereby reduce the potential immunogenicity of the antibody. This approach is also referred to as "deimmunization" and is described in further detail in U.S. Patent Publication No. 20030153043.

[0078] In addition or alternative to modifications made within the framework regions, antibodies can be engineered to include modifications within the Fc region, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody can be chemically modified (*e.g.,* one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody. Each of these embodiments is described in further detail below. The numbering of residues in the Fc region is that of the EU index of Kabat.

[0079] In a preferred embodiment, the antibody is an IgG4 isotype antibody comprising a Serine to Proline mutation at a position corresponding to position 228 (S228P; EU index) in the hinge region of the heavy chain constant region. This mutation has been reported to abolish the heterogeneity of inter-heavy chain disulfide bridges in the hinge region (Angal *et al. supra*; position 241 is based on the Kabat numbering system).

[0080] In one embodiment, the hinge region of CH1 is modified such that the number of

cysteine residues in the hinge region is altered, *e.g.,* increased or decreased. This approach is described further in U.S. Patent No. 5,677,425. The number of cysteine residues in the hinge region of CH1 is altered to, for example, facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.

[0081] In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half life of the antibody. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcyl protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent No. 6,165,745.

[0082] In another embodiment, the antibody is modified to increase its biological half life. Various approaches are possible. For example, one or more of the following mutations can be introduced: T252L, T254S, T256F, as described in U.S. Patent No. 6,277,375. Alternatively, to increase the biological half life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022.

[0083] In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector function(s) of the antibody. For example, one or more amino acids selected from amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322 can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Patent Nos. 5,624,821 and 5,648,260.

[0084] In another example, one or more amino acids selected from amino acid residues 329, 331 and 322 can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent No. 6,194,551.

[0085] In another example, one or more amino acid residues within amino acid positions 231 and 239 are altered to thereby alter the ability of the antibody to fix complement. This approach is described further in PCT Publication WO 94/29351.

[0086] In yet another example, the Fc region is modified to increase the ability of the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fcy receptor by modifying one or more amino acids at the following positions: 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439. This approach is described further in PCT Publication WO 00/42072. Moreover, the binding sites on human IgG1 for

FcyR1, FcyRII, FcyRIII and FcRn have been mapped and variants with improved binding have been described *(see* Shields et al. (2001) J. Biol. Chem. 276:6591-6604). Specific mutations at positions 256, 290, 298, 333, 334 and 339 were shown to improve binding to FcyRIII. Additionally, the following combination mutants were shown to improve FcyRIII binding: T256A/S298A, S298A/E333A, S298A/K224A and S298A/E333A/K334A.

[0087] In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycosylated antibody can be made (*i.e.,* the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. *See, e.g.,* U.S. Patent Nos. 5,714,350 and 6,350,861.

[0088] Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies to thereby produce an antibody with altered glycosylation. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (α (1,6)-fucosyltransferase), such that antibodies expressed in the Ms704, Ms705, and Ms709 cell lines lack fucose on their carbohydrates. The Ms704, Ms705, and Ms709 $FUT8^{-/-}$ cell lines were created by the targeted disruption of the FUT8 gene in CHO/DG44 cells using two replacement vectors (*see* U.S. Patent Publication No. 20040110704 and Yamane-Ohnuki et al. (2004) Biotechnol Bioeng 87:614-22). As another example, EP 1,176,195 describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation by reducing or eliminating the α-1,6 bond-related enzyme. EP 1,176,195 also describes cell lines which have a low enzyme activity for adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity, for example the rat myeloma cell line YB2/0 (ATCC CRL 1662). PCT Publication WO 03/035835 describes a variant CHO cell line, Lec13 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (*see* also Shields et al. (2002) J. Biol. Chem. 277:26733-26740). Antibodies with a modified glycosylation profile can also be produced in chicken eggs, as described in PCT Publication WO 06/089231. Alternatively, antibodies with a modified glycosylation profile can be produced in plant cells, such as *Lemna.* Methods for production of antibodies in a plant system are disclosed in the U.S. Patent application corresponding to Alston & Bird LLP attorney docket No. 040989/314911, filed on August 11, 2006. PCT Publication WO 99/54342 describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.,* β(1,4)-N-acetylglucosaminyltransferase III

(GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies *(see* also Umana et al. (1999) Nat. Biotech. 17:176-180). Alternatively, the fucose residues of the antibody can be cleaved off using a fucosidase enzyme; *e.g.,* the fucosidase α-L-fucosidase removes fucosyl residues from antibodies (Tarentino et al. (1975) Biochem. 14:5516-23).

[0089] Another modification of the antibodies herein that is contemplated by this disclosure is pegylation. An antibody can be pegylated to, for example, increase the biological (*e.g.,* serum) half-life of the antibody. To pegylate an antibody, the antibody, or fragment thereof, typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. In certain embodiments, the antibody to be pegylated is an aglycosylated antibody. Methods for pegylating proteins are known in the art and can be applied to the antibodies for use in the invention. *See, e.g.,* EP 0 154 316 and EP 0 401 384.

Antibody Physical Properties

[0090] Antibodies can be characterized by their various physical properties, to detect and/or differentiate different classes thereof.

[0091] For example, antibodies can contain one or more glycosylation sites in either the light or heavy chain variable region. Such glycosylation sites may result in increased immunogenicity of the antibody or an alteration of the pK of the antibody due to altered antigen binding (Marshall et al (1972) Annu Rev Biochem 41:673-702; Gala and Morrison (2004) J Immunol 172:5489-94; Wallick et al (1988) J Exp Med 168:1099-109; Spiro (2002) Glycobiology 12:43R-56R; Parekh et al (1985) Nature 316:452-7; Mimura et al. (2000) Mol Immunol 37:697-706). Glycosylation has been known to occur at motifs containing an N-X-S/T sequence. In some instances, it is preferred to have an anti-LAG-3 antibody that does not contain variable region glycosylation. This can be achieved either by selecting antibodies that do not contain the glycosylation motif in the variable region or by mutating residues within the glycosylation region.

[0092] In a preferred embodiment, the antibodies do not contain asparagine isomerism sites. The deamidation of asparagine may occur on N-G or D-G sequences and result in the creation of an isoaspartic acid residue that introduces a kink into the polypeptide chain and decreases its stability (isoaspartic acid effect).

[0093] Each antibody will have a unique isoelectric point (pI), which generally falls in the pH

range between 6 and 9.5. The pI for an IgG1 antibody typically falls within the pH range of 7-9.5 and the pI for an IgG4 antibody typically falls within the pH range of 6-8. There is speculation that antibodies with a pI outside the normal range may have some unfolding and instability under *in vivo* conditions. Thus, it is preferred to have an anti-LAG-3 antibody that contains a pI value that falls in the normal range. This can be achieved either by selecting antibodies with a pI in the normal range or by mutating charged surface residues.

Nucleic Acid Molecules Encoding Antibodies

[0094] In another aspect, nucleic acid molecules that encode heavy and/or light chain variable regions, or CDRs, of the antibodies for use in the invention are described. The nucleic acids can be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. *See,* Ausubel, et al., ed. (1987) Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York. A nucleic acid for use in the invention can be, *e.g.,* DNA or RNA and may or may not contain intronic sequences. It is preferred that the nucleic acid is a cDNA molecule.

[0095] Nucleic acids for use in the invention can be obtained using standard molecular biology techniques. For antibodies expressed by hybridomas (*e.g.,* hybridomas prepared from transgenic mice carrying human immunoglobulin genes as described further below), cDNAs encoding the light and heavy chains of the antibody made by the hybridoma can be obtained by standard PCR amplification or cDNA cloning techniques. For antibodies obtained from an immunoglobulin gene library (*e.g.,* using phage display techniques), a nucleic acid encoding such antibodies can be recovered from the gene library.

[0096] Preferred nucleic acids molecules for use in the invention include those encoding the V_H and V_L sequences of LAG3.5 monoclonal antibody (SEQ ID NOs: 12 and 14, respectively) or the CDRs. Once DNA fragments encoding V_H and V_L segments are obtained, these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a V_L- or V_H-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

[0097] The isolated DNA encoding the V_H region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant

region genes are known in the art (*see e.g.,* Kabat *et al.* (1991), *supra*) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the V_H-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

[0098] The isolated DNA encoding the V_L region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the V_L-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (*see e.g.,* Kabat *et al., supra*) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. It is preferred that the light chain constant region can be a kappa or lambda constant region.

[0099] To create a scFv gene, the V_H- and V_L-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, e.g., encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$ (SEQ ID NO: 28), such that the V_H and V_L sequences can be expressed as a contiguous single-chain protein, with the V_L and V_H regions joined by the flexible linker *(see e.g.,* Bird et al. (1988) Science 242:423-426; Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883; McCafferty et al., (1990) Nature 348:552-554).

Production of Monoclonal Antibodies

[0100] Monoclonal antibodies (mAbs) for use in the present invention can be produced using the well-known somatic cell hybridization (hybridoma) technique of Kohler and Milstein (1975) Nature 256: 495. Other ways for producing monoclonal antibodies include viral or oncogenic transformation of B lymphocytes and phage display techniques. Chimeric or humanized antibodies are also well known in the art. *See* e.g., U.S. Patent Nos. 4,816,567; 5,225,539; 5,530,101; 5,585,089; 5,693,762 and 6,180,370.

[0101] It is preferred that the antibodies are human monoclonal antibodies. Such human monoclonal antibodies directed against human LAG-3 can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include mice referred to herein as the HuMAb Mouse® and KM Mouse®, respectively, and are collectively referred to herein as "human Ig mice."

[0102] The HuMAb Mouse® (Medarex®, Inc.) contains human immunoglobulin gene miniloci that encode unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci *(see e.g.,* Lonberg et al. (1994) Nature 368(6474): 856-859). Accordingly, the mice exhibit

reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ monoclonal antibodies (Lonberg *et al.* (1994), *supra;* reviewed in Lonberg (1994) Handbook of Experimental Pharmacology 113:49-101; Lonberg, N. and Huszar, D. (1995) Intern. Rev. Immunol. 13: 65-93, and Harding and Lonberg (1995) Ann. N.Y. Acad. Sci. 764:536-546). Preparation and use of the HuMAb Mouse®, and the genomic modifications carried by such mice, is further described in Taylor et al. (1992) Nucleic Acids Research 20:6287-6295; Chen et al. (1993) International Immunology 5: 647-656; Tuaillon et al. (1993) Proc. Natl. Acad. Sci. USA 90:3720-3724; Choi et al. (1993) Nature Genetics 4:117-123; Chen et al. (1993) EMBO J. 12: 821-830; Tuaillon et al. (1994) J. Immunol. 152:2912-2920; Taylor et al. (1994) International Immunology 6: 579-591; and Fishwild et al. (1996) Nature Biotechnology 14: 845-851. *See* further, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; 5,770,429; and 5,545,807; PCT Publication Nos. WO 92/03918; WO 93/12227; WO 94/25585; WO 97/13852; WO 98/24884; WO 99/45962 and WO 01/14424.

[0103] Human antibodies can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchomosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. This mouse is referred to herein as a "KM mouse®," and is described in detail in PCT Publication WO 02/43478. A modified form of this mouse, which further comprises a homozygous disruption of the endogenous FcγRIIB receptor gene, is also described in PCT Publication WO 02/43478 and referred to herein as a "KM/FCGR2D mouse®." In addition, mice with either the HCo7 or HCo12 heavy chain transgenes or both can be used.

[0104] Additional transgenic animals include the Xenomouse (Abgenix, Inc., U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963). Further animals include "TC mice" (Tomizuka et al. (2000) Proc. Natl. Acad. Sci. USA 97:722-727) and cows carrying human heavy and light chain transchromosomes (Kuroiwa et al. (2002) Nature Biotechnology 20:889-894; PCT Publication WO 02/092812).

[0105] Human monoclonal antibodies can be prepared using phage display methods for screening libraries of human immunoglobulin genes. *See, e.g.* U.S. Patent Nos. 5,223,409; 5,403,484; 5,571,698; 5,427,908; 5,580,717; 5,969,108;6,172,197; 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915; and 6,593,081.

[0106] Human monoclonal antibodies can also be prepared using SCID mice into which human immune cells have been reconstituted such that a human antibody response can be generated upon immunization. *See, e.g.,* U.S. Patent Nos. 5,476,996 and 5,698,767.

[0107] Human anti-LAG-3 antibodies can also be prepared using phage display where the phages comprise nucleic acids encoding antibodies generated in transgenic animals previously immunized with LAG-3. It is preferred that the transgenic animal is a HuMab, KM, or Kirin

mouse. *See, e.g.* U.S. Patent No. 6,794,132.

Immunization of Human Ig Mice

[0108] In one example, human Ig mice are immunized with a purified or enriched preparation of a LAG-3 antigen, recombinant LAG-3 protein, or cells expressing a LAG-3 protein. *See, e.g.,* Lonberg *et al.* (1994), *supra;* Fishwild *et al.* (1996), *supra;* PCT Publications WO 98/24884 or WO 01/14424. It is preferred that 6-16 week old mice are immunized with 5-50 µg of LAG-3 protein. Alternatively, a portion of LAG-3 fused to a non-LAG-3 polypeptide is used.

[0109] The transgenic mice can be immunized intraperitoneally (IP) or intravenously (IV) with LAG-3 antigen in complete Freund's adjuvant, followed by subsequent IP or IV immunizations with antigen in incomplete Freund's adjuvant. Adjuvants other than Freund's or whole cells in the absence of adjuvant can also be used. The plasma can be screened by ELISA and cells from mice with sufficient titers of anti-LAG-3 human immunoglobulin can be used for fusions.

Generation of Hybridomas Producing Human Monoclonal Antibodies

[0110] To generate hybridomas producing human monoclonal antibodies for use in the invention, splenocytes and/or lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can be screened for the production of antigen-specific antibodies. Generation of hybridomas is well-known in the art. *See, e.g.,* Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York.

Generation of Transfectomas Producing Monoclonal Antibodies

[0111] Antibodies for use in the invention also can be produced in a host cell transfectoma using, for example, a combination of recombinant DNA techniques and gene transfection methods as is well known in the art (*e.g.,* Morrison, S. (1985) Science 229:1202). DNA encoding partial or full-length light and heavy chains obtained by standard molecular biology techniques can be inserted into one or more expression vectors such that the genes are operatively linked to transcriptional and translational regulatory sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene.

[0112] The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.,* polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, *e.g.,* in

Goeddel (Gene Expression Technology. Methods in Enzymology 185, Academic Press, San Diego, CA (1990)). Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV), Simian Virus 40 (SV40), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP) and polyoma. Alternatively, nonviral regulatory sequences can be used, such as the ubiquitin promoter or β-globin promoter. Still further, regulatory elements composed of sequences from different sources, such as the SRα promoter system, which contains sequences from the SV40 early promoter and the long terminal repeat of human T cell leukemia virus type 1 (Takebe et al. (1988) Mol. Cell. Biol. 8:466-472). The expression vector and expression control sequences are chosen to be compatible with the expression host cell used.

[0113] The antibody light chain gene and the antibody heavy chain gene can be inserted into the same or separate expression vectors. It is preferred that the variable regions are used to create full-length antibody genes of any antibody isotype by inserting them into expression vectors already encoding heavy chain constant and light chain constant regions of the desired isotype such that the V_H segment is operatively linked to the C_H segment(s) within the vector and the V_L segment is operatively linked to the C_L segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.,* a signal peptide from a non-immunoglobulin protein).

[0114] In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors can carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.,* origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced *(see, e.g.,* U.S. Pat. Nos. 4,399,216; 4,634,665 and 5,179,017). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr-host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

[0115] For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.,* electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

[0116] Preferred mammalian host cells for expressing the recombinant antibodies include Chinese Hamster Ovary (CHO cells) (including dhfr$^-$ CHO cells, described in Urlaub and Chasin, (1980) Proc. Natl. Acad. Sci. USA 77:4216-4220, used with a DHFR selectable marker, *e.g.,* as described in R. J. Kaufman and P. A. Sharp (1982) J. Mol. Biol. 159:601-621), NSO myeloma cells, COS cells and SP2 cells. In particular, for use with NSO myeloma cells, another preferred expression system is the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338,841. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Immunoconjugates

[0117] Antibodies can be conjugated to a therapeutic agent to form an immunoconjugate such as an antibody-drug conjugate (ADC). Suitable therapeutic agents include antimetabolites, alkylating agents, DNA minor groove binders, DNA intercalators, DNA crosslinkers, histone deacetylase inhibitors, nuclear export inhibitors, proteasome inhibitors, topoisomerase I or II inhibitors, heat shock protein inhibitors, tyrosine kinase inhibitors, antibiotics, and anti-mitotic agents. In the ADC, the antibody and therapeutic agent preferably are conjugated via a linker cleavable such as a peptidyl, disulfide, or hydrazone linker. More preferably, the linker is a peptidyl linker such as Val-Cit, Ala-Val, Val-Ala-Val, Lys-Lys, Pro-Val-Gly-Val-Val (SEQ ID NO: 39), Ala-Asn-Val, Val-Leu-Lys, Ala-Ala-Asn, Cit-Cit, Val-Lys, Lys, Cit, Ser, or Glu. The ADCs can be prepared as described in U.S. Patent Nos. 7,087,600; 6,989,452; and 7,129,261; PCT Publications WO 02/096910; WO 07/038658; WO 07/051081; WO 07/059404; WO 08/083312; and WO 08/103693; U.S. Patent Publications 20060024317; 20060004081; and 20060247295.

Bispecific Molecules

[0118] In another aspect, the present disclosure features bispecific molecules comprising one or more antibodies linked to at least one other functional molecule, e.g., another peptide or protein (e.g., another antibody or ligand for a receptor) to generate a bispecific molecule that binds to at least two different binding sites or target molecules. Thus, as used herein, "bispecific molecule" includes molecules that have three or more specificities. It is preferred that the bispecific molecule comprises a first binding specificity for LAG-3 and a second binding specificity for a triggering molecule that recruits cytotoxic effector cells that can kill a LAG-3 expressing target cell. Examples of suitable triggering molecules are CD64, CD89, CD16, and CD3. *See, e.g.,* Kufer et al., TRENDS in Biotechnology, 22 (5), 238-244 (2004).

[0119] A bispecific molecule can have, in addition to an anti-Fc binding specificity and an anti-LAG-3 binding specificity, a third specificity. The third specificity can be for an anti-enhancement factor (EF), *e.g.,* a molecule that binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. For example, the anti-enhancement factor can bind a cytotoxic T-cell (*e.g.* via CD2, CD3, CD8, CD28, CD4, CD40, or ICAM-1) or other immune cell, resulting in an increased immune response against the target cell.

[0120] Bispecific molecules can come in many different formats and sizes. At one end of the size spectrum, a bispecific molecule retains the traditional antibody format, except that, instead of having two binding arms of identical specificity, it has two binding arms each having a different specificity. At the other extreme are bispecific molecules consisting of two single-chain antibody fragments (scFv's) linked by a peptide chain, a so-called $Bs(scFv)_2$ construct. Intermediate-sized bispecific molecules include two different F(ab) fragments linked by a peptidyl linker. Bispecific molecules of these and other formats can be prepared by genetic engineering, somatic hybridization, or chemical methods. *See, e.g.,* Kufer *et al,* cited *supra;* Cao and Suresh, Bioconjugate Chemistry, 9 (6), 635-644 (1998); and van Spriel et al., Immunology Today, 21 (8), 391-397 (2000), and the references cited therein.

Pharmaceutical Compositions

[0121] In another aspect, the present disclosure provides a pharmaceutical composition comprising one or more antibodies formulated together with a pharmaceutically acceptable carrier. The composition may optionally contain one or more additional pharmaceutically active ingredients, such as another antibody or a drug. The pharmaceutical compositions of the invention also can be administered in a combination therapy with, for example, another immunostimulatory agent, anti-cancer agent, an anti-viral agent, or a vaccine, such that the anti-LAG-3 antibody enhances the immune response against the vaccine.

[0122] The pharmaceutical composition can comprise any number of excipients. Excipients that can be used include carriers, surface active agents, thickening or emulsifying agents, solid binders, dispersion or suspension aids, solubilizers, colorants, flavoring agents, coatings, disintegrating agents, lubricants, sweeteners, preservatives, isotonic agents, and combinations thereof. The selection and use of suitable excipients is taught in Gennaro, ed., Remington: The Science and Practice of Pharmacy, 20th Ed. (Lippincott Williams & Wilkins 2003).

[0123] Preferably, the pharmaceutical composition is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.,* by injection or infusion). Depending on the route of administration, the active compound can be coated in a material to protect it from the action of acids and other natural conditions that may inactivate it. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac,

intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Alternatively, an antibody can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, *e.g.,* intranasally, orally, vaginally, rectally, sublingually or topically.

[0124] The pharmaceutical compositions of the invention can include pharmaceutically acceptable salts. A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects. Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

[0125] Pharmaceutical compositions can be in the form of sterile aqueous solutions or dispersions. They can also be formulated in a microemulsion, liposome, or other ordered structure suitable to high drug concentration.

[0126] The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated and the particular mode of administration and will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 0.01% to about ninety-nine percent of active ingredient, preferably from about 0.1% to about 70%, most preferably from about 1% to about 30% of active ingredient in combination with a pharmaceutically acceptable carrier.

[0127] Dosage regimens are adjusted to provide the optimum desired response (*e.g.,* a therapeutic response). For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required.

[0128] For administration of the antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 0.3

mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months. Preferred dosage regimens for an anti-LAG-3 antibody include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the antibody being given using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks. In some methods, dosage is adjusted to achieve a plasma antibody concentration of about 1-1000 µg /ml and in some methods about 25-300 µg /ml.

[0129] A "therapeutically effective dosage" of an anti-LAG-3 antibody preferably results in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. For example, for the treatment of tumor-bearing subjects, a "therapeutically effective dosage" preferably inhibits tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject, which is typically a human or can be another mammal.

[0130] The pharmaceutical composition can be a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. *See, e.g.,* Sustained and Controlled Release Drug Delivery Systems, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

[0131] Therapeutic compositions can be administered via medical devices such as (1) needleless hypodermic injection devices (e.g., US 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; and 4,596,556); (2) micro-infusion pumps (US 4,487,603); (3) transdermal devices (US 4,486,194); (4) infusion apparati (US 4,447,233 and 4,447,224); and (5) osmotic devices (US 4,439,196 and 4,475,196).

[0132] The human monoclonal antibodies can be formulated to ensure proper distribution *in vivo.* For example, to ensure that the therapeutic compounds cross the blood-brain barrier, they can be formulated in liposomes, which may additionally comprise targeting moieties to enhance selective transport to specific cells or organs. *See, e.g.* US 4,522,811; 5,374,548; 5,416,016; and 5,399,331; V.V. Ranade (1989) J. Clin. Pharmacol. 29:685; Umezawa et al., (1988) Biochem. Biophys. Res. Commun. 153:1038; Bloeman et al. (1995) FEBS Lett. 357:140; M. Owais et al. (1995) Antimicrob. Agents Chemother. 39:180; Briscoe et al. (1995) Am. J. Physiol. 1233:134; Schreier et al. (1994) J. Biol. Chem. 269:9090; Keinanen and Laukkanen (1994) FEBS Lett. 346:123; and Killion and Fidler (1994) Immunomethods 4:273.

Uses of the Invention

[0133] Compositions, of the present invention have numerous *in vitro* and *in vivo* utilities involving, for example, detection of LAG-3 or enhancement of immune responses by blockade of LAG-3. It is preferred that the antibodies are human antibodies. Such antibodies can be administered to cells in culture, *in vitro* or *ex vivo,* or to human subjects, *e.g., in vivo,* to enhance immunity in a variety of situations. Accordingly, in one aspect, the disclosure provides a method of modifying an immune response in a subject comprising administering to the subject the composition of the invention such that the immune response in the subject is modified. Preferably, the response is enhanced, stimulated or up-regulated.

[0134] Preferred subjects include human patients in need of enhancement of an immune response. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting an immune response (*e.g.,* the T-cell mediated immune response). The methods are particularly suitable for treatment of cancer *in vivo.* To achieve antigen-specific enhancement of immunity, the anti-LAG-3 antibodies can be administered together with an antigen of interest or the antigen may already be present in the subject to be treated (*e.g.,* a tumor-bearing or virus-bearing subject). When antibodies to LAG-3 are administered together with another agent, the two can be administered in either order or simultaneously.

[0135] Also described herein are methods for detecting the presence of human LAG-3 antigen in a sample, or measuring the amount of human LAG-3 antigen, comprising contacting the sample, and a control sample, with a human monoclonal antibody, or an antigen binding portion thereof, which specifically binds to human LAG-3, under conditions that allow for formation of a complex between the antibody or portion thereof and human LAG-3. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of human LAG-3 antigen in the sample. Moreover, the anti-LAG-3 antibodies of the invention can be used to purify human LAG-3 via immunoaffinity purification.

[0136] Given the ability of anti-LAG-3 antibodies of the invention to inhibit the binding of LAG-3 to MHC Class II molecules and to stimulate antigen-specific T cell responses, the compositions of the invention can also be used in *in vitro* and *in vivo* methods to stimulate, enhance or upregulate antigen-specific T cell responses. For example, the invention provides compositions for use in a method of stimulating an antigen-specific T cell response comprising contacting said T cell with a composition of the invention, such that an antigen-specific T cell response is stimulated. Any suitable indicator of an antigen-specific T cell response can be used to measure the antigen-specific T cell response. Non-limiting examples of such suitable indicators include increased T cell proliferation in the presence of the antibody and/or increase cytokine production in the presence of the antibody. In a preferred embodiment, interleukin-2 production by the antigen-specific T cell is stimulated.

[0137] The invention also provides compositions for use in a method of stimulating an immune response (*e.g.,* an antigen-specific T cell response) in a subject comprising administering a composition of the invention to the subject such that an immune response (*e.g.,* an antigen-specific T cell response) in the subject is stimulated. In a preferred embodiment, the subject is a tumor-bearing subject and an immune response against the tumor is stimulated. In another preferred embodiment, the subject is a virus-bearing subject and an immune response against the virus is stimulated.

[0138] In another embodiment, the composition is for use in methods for inhibiting growth of tumor cells in a subject comprising administering to the subject a composition of the invention such that growth of the tumor is inhibited in the subject. In yet another embodiment, the composition is for use in methods for treating a viral infection in a subject comprising administering to the subject an antibody of the invention such that the viral infection is treated in the subject.

[0139] These and other uses are discussed in further detail below.

Cancer

[0140] Blockade of LAG-3 by antibodies can enhance the immune response to cancerous cells in the patient. In one aspect, the present invention relates to treatment of a subject *in vivo* using an anti-LAG-3 antibody such that growth of cancerous tumors is inhibited. An anti-LAG-3 antibody can be used alone to inhibit the growth of cancerous tumors. Alternatively, an anti-LAG-3 antibody can be used in conjunction with other immunogenic agents, standard cancer treatments, or other antibodies, as described below.

[0141] Accordingly, in one embodiment, the composition is for use in a method of inhibiting growth of tumor cells in a subject, comprising administering to the subject a therapeutically effective amount of an anti-LAG-3 antibody, or antigen-binding portion thereof. Preferably, the antibody is a human anti-LAG-3 antibody (such as any of the human anti-human LAG-3 antibodies described herein). Additionally or alternatively, the antibody can be a chimeric or humanized anti-LAG-3 antibody.

[0142] Preferred cancers whose growth may be inhibited using the antibodies of the invention include cancers typically responsive to immunotherapy. Non-limiting examples of preferred cancers for treatment include melanoma (*e.g.,* metastatic malignant melanoma), renal cancer (*e.g.* clear cell carcinoma), prostate cancer (*e.g.* hormone refractory prostate adenocarcinoma), breast cancer, colon cancer and lung cancer (*e.g.* non-small cell lung cancer). Additionally, the invention includes refractory or recurrent malignancies whose growth may be inhibited using the antibodies of the invention.

[0143] Examples of other cancers that can be treated using the compositions of the invention

include bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced cancers including those induced by asbestos, and combinations of said cancers. The present invention is also useful for treatment of metastatic cancers, especially metastatic cancers that express PD-L1 (Iwai et al. (2005) Int. Immunol. 17:133-144).

[0144] Optionally, antibodies to LAG-3 can be combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells, and cells transfected with genes encoding immune stimulating cytokines (He et al (2004) J. Immunol. 173:4919-28). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gp100, MAGE antigens, Trp-2, MART1 and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF (discussed further below).

[0145] In humans, some tumors have been shown to be immunogenic such as melanomas. By raising the threshold of T cell activation by LAG-3 blockade, the tumor responses in the host can be activated.

[0146] LAG-3 blockade is likely to be more effective when combined with a vaccination protocol. Many experimental strategies for vaccination against tumors have been devised (*see* Rosenberg, S., 2000, Development of Cancer Vaccines, ASCO Educational Book Spring: 60-62; Logothetis, C., 2000, ASCO Educational Book Spring: 300-302; Khayat, D. 2000, ASCO Educational Book Spring: 414-428; Foon, K. 2000, ASCO Educational Book Spring: 730-738; *see* also Restifo, N. and Sznol, M., Cancer Vaccines, Ch. 61, pp. 3023-3043 in DeVita et al. (eds.), 1997, Cancer: Principles and Practice of Oncology, Fifth Edition). In one of these strategies, a vaccine is prepared using autologous or allogeneic tumor cells. These cellular vaccines have been shown to be most effective when the tumor cells are transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff et al. (1993) Proc. Natl. Acad. Sci U.S.A. 90: 3539-43).

[0147] The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg, SA (1999) Immunity 10: 281-7). In many cases, these tumor specific antigens are differentiation antigens

expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE antigens, and Trp-2. More importantly, many of these antigens can be shown to be the targets of tumor specific T cells found in the host. LAG-3 blockade can be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. These proteins are normally viewed by the immune system as self antigens and are therefore tolerant to them. The tumor antigen can include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim et al. (1994) Science 266: 2011-2013). (These somatic tissues may be protected from immune attack by various means). Tumor antigen can also be "neo-antigens" expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (*i.e.,* bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

[0148] Other tumor vaccines can include the proteins from viruses implicated in human cancers such a Human Papilloma Viruses (HPV), Hepatitis Viruses (HBV and HCV) and Kaposi's Herpes Sarcoma Virus (KHSV). Another form of tumor specific antigen which can be used in conjunction with LAG-3 blockade is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat shock proteins contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity (Suot & Srivastava (1995) Science 269:1585-1588; Tamura et al. (1997) Science 278:117-120).

[0149] Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC's can be produced *ex vivo* and loaded with various protein and peptide antigens as well as tumor cell extracts (Nestle et al. (1998) Nature Medicine 4: 328-332). DCs can also be transduced by genetic means to express these tumor antigens as well. DCs have also been fused directly to tumor cells for the purposes of immunization (Kugler et al. (2000) Nature Medicine 6:332-336). As a method of vaccination, DC immunization can be effectively combined with LAG-3 blockade to activate more potent anti-tumor responses.

[0150] LAG-3 blockade can also be combined with standard cancer treatments. LAG-3 blockade can be effectively combined with chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered (Mokyr et al. (1998) Cancer Research 58: 5301-5304). An example of such a combination is an anti-LAG-3 antibody in combination with decarbazine for the treatment of melanoma. Another example of such a combination is an anti-LAG-3 antibody in combination with interleukin-2 (IL-2) for the treatment of melanoma. The scientific rationale behind the combined use of LAG-3 blockade and chemotherapy is that cell death, that is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with LAG-3 blockade through cell death are radiation, surgery, and hormone deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors can also be combined with LAG-3 blockade. Inhibition of angiogenesis leads to tumor cell death which may

feed tumor antigen into host antigen presentation pathways.

[0151] LAG-3 blocking antibodies can also be used in combination with bispecific antibodies that target Fcα or Fcγ receptor-expressing effectors cells to tumor cells (*see, e.g.,* U.S. Pat. Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. For example anti-Fc receptor/anti tumor antigen (*e.g.,* Her-2/neu) bispecific antibodies have been used to target macrophages to sites of tumor. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the use of LAG-3 blockade. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies which bind to tumor antigen and a dendritic cell specific cell surface marker.

[0152] Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of proteins which are expressed by the tumors and which are immunosuppressive. These include among others TGF-β (Kehrl et al. (1986) J. Exp. Med. 163: 1037-1050), IL-10 (Howard & O'Garra (1992) Immunology Today 13: 198-200), and Fas ligand (Hahne et al. (1996) Science 274: 1363-1365). Antibodies to each of these entities can be used in combination with anti-LAG-3 to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

[0153] Other antibodies which activate host immune responsiveness can be used in combination with anti-LAG-3. These include molecules on the surface of dendritic cells which activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity (Ridge et al. (1998) Nature 393: 474-478) and can be used in conjunction with LAG-3 antibodies (Ito et al. (2000) Immunobiology 201 (5) 527-40). Activating antibodies to T cell costimulatory molecules such as CTLA-4 (*e.g.,* US Patent No. 5,811,097), OX-40 (Weinberg et al. (2000) Immunol 164: 2160-2169), 4-1BB (Melero et al. (1997) Nature Medicine 3: 682-685 (1997), and ICOS (Hutloff et al. (1999) Nature 397: 262-266) may also provide for increased levels of T cell activation.

[0154] Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, therapeutic benefit may be obtained from graft vs. tumor responses. LAG-3 blockade can be used to increase the effectiveness of the donor engrafted tumor specific T cells.

[0155] There are also several experimental treatment protocols that involve *ex vivo* activation and expansion of antigen specific T cells and adoptive transfer of these cells into recipients in order to stimulate antigen-specific T cells against tumor (Greenberg & Riddell (1999) Science 285: 546-51). These methods can also be used to activate T cell responses to infectious agents such as CMV. *Ex vivo* activation in the presence of anti-LAG-3 antibodies can increase the frequency and activity of the adoptively transferred T cells.

Infectious Diseases

[0156] The compositions of the invention can also be used to treat patients that have been exposed to particular toxins or pathogens. Accordingly, another aspect of the invention provides the compositions for use in a method of treating an infectious disease in a subject comprising administering to the subject the composition of the invention, such that the subject is treated for the infectious disease. Preferably, the antibody is a human anti-human LAG-3 antibody (such as any of the human anti-LAG-3 antibodies described herein). Additionally or alternatively, the antibody can be a chimeric or humanized antibody.

[0157] Similar to its application to tumors as discussed above, antibody mediated LAG-3 blockade can be used alone, or as an adjuvant, in combination with vaccines, to stimulate the immune response to pathogens, toxins, and self-antigens. Examples of pathogens for which this therapeutic approach can be particularly useful, include pathogens for which there is currently no effective vaccine, or pathogens for which conventional vaccines are less than completely effective. These include, but are not limited to HIV, Hepatitis (A, B, & C), Influenza, Herpes, Giardia, Malaria, Leishmania, Staphylococcus aureus, Pseudomonas aeruginosa. LAG-3 blockade is particularly useful against established infections by agents such as HIV that present altered antigens over the course of the infections. These novel epitopes are recognized as foreign at the time of anti-human LAG-3 administration, thus provoking a strong T cell response that is not dampened by negative signals through LAG-3.

[0158] Some examples of pathogenic viruses causing infections treatable by the compositions of the invention include HIV, hepatitis (A, B, or C), herpes virus (*e.g.,* VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, coronavirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus.

[0159] Some examples of pathogenic bacteria causing infections treatable by the compositions of the invention include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci and gonococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lymes disease bacteria.

[0160] Some examples of pathogenic fungi causing infections treatable by the compositions of the invention include Candida (albicans, krusei, glabrata, tropicalis, *etc.*), Cryptococcus neoformans, Aspergillus (fumigatus, niger, etc.), Genus Mucorales (mucor, absidia, rhizopus), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis and Histoplasma capsulatum.

[0161] Some examples of pathogenic parasites causing infections treatable by the compositions of the invention include Entamoeba histolytica, Balantidium coli, Naegleriafowleri, Acanthamoeba sp., Giardia lambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani,

Toxoplasma gondii, Nippostrongylus brasiliensis.

[0162] In all of the above methods, LAG-3 blockade can be combined with other forms of immunotherapy such as cytokine treatment (e.g., interferons, GM-CSF, G-CSF, IL-2), or bispecific antibody therapy, which provides for enhanced presentation of tumor antigens *(see, e.g.,* Holliger (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak (1994) Structure 2:1121-1123).

Autoimmune reactions

[0163] Anti-LAG-3 antibodies may provoke and amplify autoimmune responses. Indeed, induction of anti-tumor responses using tumor cell and peptide vaccines reveals that many anti-tumor responses involve anti-self reactivities (van Elsas et al. (2001) J. Exp. Med. 194:481-489; Overwijk, et al. (1999) Proc. Natl. Acad. Sci. U.S.A. 96: 2982-2987; Hurwitz, (2000) *supra;* Rosenberg & White (1996) J. Immunother Emphasis Tumor Immunol 19 (1): 81-4). Therefore, it is possible to consider using anti-LAG-3 blockade in conjunction with various self proteins in order to devise vaccination protocols to efficiently generate immune responses against these self proteins for disease treatment. For example, Alzheimer's disease involves inappropriate accumulation of Aβ peptide in amyloid deposits in the brain; antibody responses against amyloid are able to clear these amyloid deposits (Schenk et al., (1999) Nature 400: 173-177).

[0164] Other self proteins can also be used as targets such as IgE for the treatment of allergy and asthma, and TNFα for rheumatoid arthritis. Finally, antibody responses to various hormones may be induced by the use of anti-LAG-3 antibody. Neutralizing antibody responses to reproductive hormones can be used for contraception. Neutralizing antibody response to hormones and other soluble factors that are required for the growth of particular tumors can also be considered as possible vaccination targets.

[0165] Analogous methods as described above for the use of anti-LAG-3 antibody can be used for induction of therapeutic autoimmune responses to treat patients having an inappropriate accumulation of other self-antigens, such as amyloid deposits, including Aβ in Alzheimer's disease, cytokines such as TNFα, and IgE.

Vaccines

[0166] Anti-LAG-3 antibodies can be used to stimulate antigen-specific immune responses by coadministration of an anti-LAG-3 antibody with an antigen of interest (*e.g.,* a vaccine). Accordingly, in another aspect the invention the compositions are for use in a method of enhancing an immune response to an antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) an anti-LAG-3 antibody, or antigen-binding portion thereof, such

that an immune response to the antigen in the subject is enhanced. Preferably, the antibody is a human anti-human LAG-3 antibody (such as any of the human anti-LAG-3 antibodies described herein). Additionally or alternatively, the antibody can be a chimeric or humanized antibody. The antigen can be, for example, a tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen. Non-limiting examples of such antigens include those discussed in the sections above, such as the tumor antigens (or tumor vaccines) discussed above, or antigens from the viruses, bacteria or other pathogens described above.

[0167] Suitable routes of administering the antibody compositions (*e.g.,* human monoclonal antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (*e.g.,* intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the antibody composition.

[0168] As previously described, compositions of the invention can be co-administered with one or other more therapeutic agents, *e.g.,* a cytotoxic agent, a radiotoxic agent or an immunosuppressive agent. The antibody can be linked to the agent (as an immuno-complex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.,* an anti-cancer therapy, *e.g.,* radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin (adriamycin), cisplatin bleomycin sulfate, carmustine, chlorambucil, dacarbazine and cyclophosphamide hydroxyurea which, by themselves, are only effective at levels which are toxic or subtoxic to a patient. Cisplatin is intravenously administered as a 100 mg/ml dose once every four weeks and adriamycin is intravenously administered as a 60-75 mg/ml dose once every 21 days. Co-administration of the human anti-LAG-3 antibodies, or antigen binding fragments thereof, of the present invention with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such co-administration can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the antibody.

[0169] Also described are kits comprising the compositions of the invention and instructions for use. The kit can further contain at least one additional reagent, or one or more additional human antibodies. Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

Combination therapy

[0170] The claimed invention relates a single composition comprising: (a) a monoclonal antibody, or an antigen-binding portion thereof, that binds human LAG-3, (b) an anti-PD-1

antibody, or an antigen-binding portion thereof, and (c) a pharmaceutically acceptable carrier. Accordingly, the invention relates to combination therapy in which an anti-LAG-3 antibody (or antigen-binding portion thereof) of the present invention is coadministered with one or more additional antibodies that are effective in stimulating immune responses to thereby further enhance, stimulate or upregulate immune responses in a subject.

[0171] Further described herein are methods for stimulating an immune response in a subject comprising administering to the subject an anti-LAG-3 antibody and one or more additional immunostimulatory antibodies, such as an anti-PD-1 antibody, an anti-PD-L1 antibody and/or an anti-CTLA-4 antibody, such that an immune response is stimulated in the subject, for example to inhibit tumor growth or to stimulate an anti-viral response. According to the invention, the subject is administered an anti-LAG-3 antibody and an anti-PD-1 antibody. In one embodiment, the anti-LAG-3 antibody is a human antibody, such as an antibody of the disclosure. Alternatively, the anti-LAG-3 antibody can be, for example, a chimeric or humanized antibody (*e.g.,* prepared from a mouse anti-LAG-3 mAb). In another embodiment, the at least one additional immunostimulatory antibody (*i.e.,* anti-PD-1 antibody) is a human antibody. Alternatively, the at least one additional immunostimulatory antibody can be, for example, a chimeric or humanized antibody (*e.g.,* prepared from a mouse anti-PD-1).

[0172] Further, a method for treating a hyperproliferative disease (*e.g.,* cancer), comprising administering a LAG-3 antibody and a CTLA-4 antibody to a subject, is described herein. The anti-LAG-3 antibody can be administered at a subtherapeutic dose, the anti-CTLA-4 antibody can be administered at a subtherapeutic dose, or both can be administered at a subtherapeutic dose. Further, a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering an anti-LAG-3 antibody and a subtherapeutic dose of anti-CTLA-4 antibody to a subject, is described herein. The subject may be human. The anti-CTLA-4 antibody may be human sequence monoclonal antibody 10D1 (described in PCT Publication WO 01/14424) and the anti-LAG-3 antibody may be human sequence monoclonal antibody, such as LAG3.5 described herein. Other anti-CTLA-4 antibodies encompassed by the methods described herein include, for example, those disclosed in: WO 98/42752; WO 00/37504; U.S. Patent No. 6,207,156; Hurwitz et al. (1998) Proc. Natl. Acad. Sci. USA 95(17): 10067-10071; Camacho et al. (2004) J. Clin. Oncology 22(145): Abstract No. 2505 (antibody CP-675206); and Mokyr et al. (1998) Cancer Res. 58:5301-5304. The anti-CTLA-4 antibody may bind to human CTLA-4 with a K_D of 5×10^{-8} M or less, bind to human CTLA-4 with a K_D of 1×10^{-8} M or less, bind to human CTLA-4 with a K_D of 5×10^{-9} M or less, or bind to human CTLA-4 with a K_D of between 1×10^{-8} M and 1×10^{-10} M or less.

[0173] The present invention provides compositions for use in a method for treating a hyperproliferative disease (*e.g.,* cancer), comprising administering a LAG-3 antibody and a PD-1 antibody to a subject. In further embodiments, the anti-LAG-3 antibody is administered at a subtherapeutic dose, the anti-PD-1 antibody is administered at a subtherapeutic dose, or both are administered at a subtherapeutic dose. In another embodiment, the present invention

provides compositions for use in a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering an anti-LAG-3 antibody and a subtherapeutic dose of anti-PD-1 antibody to a subject. In certain embodiments, the subject is human. In certain embodiments, the anti-PD-1 antibody is a human sequence monoclonal antibody and the anti-LAG-3 antibody is human sequence monoclonal antibody, such as LAG3.5 described herein. Examples of human sequence anti-PD-1 antibodies include 17D8, 2D3, 4H1, 5C4 and 4A11, which are described in PCT Publication WO 06/121168. Other anti-PD-1 antibodies include, e.g., lambrolizumab (WO2008/156712), and AMP514 (WO2010/027423, WO2010/027827, WO2010/027828, WO2010/098788). In certain embodiments, the anti-PD-1 antibody binds to human PD-1 with a K_D of 5×10^{-8} M or less, binds to human PD-1 with a K_D of 1×10^{-8} M or less, binds to human PD-1 with a K_D of 5×10^{-9} M or less, or binds to human PD-1 with a K_D of between 1×10^{-8} M and 1×10^{-10} M or less.

[0174] Further, a method for treating a hyperproliferative disease (*e.g.,* cancer), comprising administering a LAG-3 antibody and a PD-L1 antibody to a subject, is described herein. The anti-LAG-3 antibody may be administered at a subtherapeutic dose, the anti-PD-L1 antibody may be administered at a subtherapeutic dose, or both may be administered at a subtherapeutic dose. Further, a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering an anti-LAG-3 antibody and a subtherapeutic dose of anti-PD-L1 antibody to a subject, is described herein. The subject may be human. The anti-PD-L1 antibody may be a human sequence monoclonal antibody and the anti-LAG-3 antibody may be a human sequence monoclonal antibody, such as LAG3.5 described herein. Examples of human sequence anti-PD-L1 antibodies include 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 and 13G4, which are described in PCT Publication WO 07/005874. Other anti-PD-L1 antibodies include, e.g., MPDL3280A (RG7446) (WO2010/077634), MEDI4736 (WO2011/066389), and MDX1105 (WO2007/005874). The anti-PD-L1 antibody may bind to human PD-L1 with a K_D of 5×10^{-8} M or less, bind to human PD-L1 with a K_D of 1×10^{-8} M or less, bind to human PD-L1 with a K_D of 5×10^{-9} M or less, or bind to human PD-L1 with a K_D of between 1×10^{-8} M and 1×10^{-10} M or less.

[0175] Blockade of LAG-3 and one or more second target antigens such as CTLA-4 and/or PD-1 and/or PD-L1 by antibodies can enhance the immune response to cancerous cells in the patient. Cancers whose growth may be inhibited using the antibodies of the instant disclosure include cancers typically responsive to immunotherapy. Representative examples of cancers for treatment with the combination therapy of the instant disclosure include those cancers specifically listed above in the discussion of monotherapy with anti-LAG-3 antibodies.

[0176] The combination of therapeutic antibodies discussed herein can be administered concurrently as a single composition in a pharmaceutically acceptable carrier, or concurrently as separate compositions with each antibody in a pharmaceutically acceptable carrier. Further,

the combination of therapeutic antibodies can be administered sequentially. For example, an anti-CTLA-4 antibody and an anti-LAG-3 antibody can be administered sequentially, such as anti-CTLA-4 antibody being administered first and anti-LAG-3 antibody second, or anti-LAG-3 antibody being administered first and anti-CTLA-4 antibody second. Additionally or alternatively, an anti-PD-1 antibody and an anti-LAG-3 antibody can be administered sequentially, such as anti-PD-1 antibody being administered first and anti-LAG-3 antibody second, or anti-LAG-3 antibody being administered first and anti-PD-1 antibody second. Additionally or alternatively, an anti-PD-L1 antibody and an anti-LAG-3 antibody can be administered sequentially, such as anti-PD-L1 antibody being administered first and anti-LAG-3 antibody second, or anti-LAG-3 antibody being administered first and anti-PD-L1 antibody second. According to the claimed invention, the anti-LAG-3 antibody and the anti-PD-1 antibody are administered concurrently as a single composition in a pharmaceutically acceptable carrier.

[0177] Furthermore, if more than one dose of the combination therapy is administered sequentially, the order of the sequential administration can be reversed or kept in the same order at each time point of administration, sequential administrations can be combined with concurrent administrations, or any combination thereof. For example, the first administration of a combination anti-CTLA-4 antibody and anti-LAG-3 antibody can be concurrent, the second administration can be sequential with anti-CTLA-4 first and anti-LAG-3 second, and the third administration can be sequential with anti-LAG-3 first and anti-CTLA-4 second, *etc.* Additionally or alternatively, the first administration of a combination anti-PD-1 antibody and anti-LAG-3 antibody can be concurrent, the second administration can be sequential with anti-PD-1 first and anti-LAG-3 second, and the third administration can be sequential with anti-LAG-3 first and anti-PD-1 second, etc. Additionally or alternatively, the first administration of a combination anti-PD-L1 antibody and anti-LAG-3 antibody can be concurrent, the second administration can be sequential with anti-PD-L1 first and anti-LAG-3 second, and the third administration can be sequential with anti-LAG-3 first and anti-PD-L1 second, *etc.* Another representative dosing scheme can involve a first administration that is sequential with anti-LAG-3 first and anti-CTLA-4 (and/or anti-PD-1 and/or anti-PD-L1) second, and subsequent administrations may be concurrent.

[0178] Optionally, the combination of anti-LAG-3 and one or more additional antibodies (*e.g.,* anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibodies) can be further combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells, and cells transfected with genes encoding immune stimulating cytokines (He et al. (2004) J. Immunol. 173:4919-28). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gp100, MAGE antigens, Trp-2, MART1 and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF (discussed further below). A combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade can be further combined with a vaccination protocol, such as any of the vaccination protocols discussed in detail above with respect to monotherapy with anti-LAG-3 antibodies.

[0179] A combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade can also be further combined with standard cancer treatments. For example, a combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade can be effectively combined with chemotherapeutic regimes. In these instances, it is possible to reduce the dose of other chemotherapeutic reagent administered with the combination of the instant disclosure (Mokyr et al. (1998) Cancer Research 58: 5301-5304). An example of such a combination is a combination of anti-LAG-3 and anti-CTLA-4 antibodies and/or anti-PD-1 antibodies and/or anti-PD-L1 antibodies further in combination with decarbazine for the treatment of melanoma. Another example is a combination of anti-LAG-3 and anti-CTLA-4 antibodies and/or anti-PD-1 antibodies and/or anti-PD-L1 antibodies further in combination with interleukin-2 (IL-2) for the treatment of melanoma. The scientific rationale behind the combined use of LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade with chemotherapy is that cell death, which is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with a combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade through cell death include radiation, surgery, or hormone deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors can also be combined with a combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade. Inhibition of angiogenesis leads to tumor cell death, which can be a source of tumor antigen fed into host antigen presentation pathways.

[0180] A combination of LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blocking antibodies can also be used in combination with bispecific antibodies that target Fcα or Fcγ receptor-expressing effector cells to tumor cells (*see, e.g.,* U.S. Pat. Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. The T cell arm of these responses would be augmented by the use of a combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade.

[0181] In another example, a combination of anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 antibodies and/or anti-PD-L1 antibodies can be used in conjunction with anti-neoplastic antibodies, such as Rituxan® (rituximab), Herceptin® (trastuzumab), Bexxar® (tositumomab), Zevalin® (ibritumomab), Campath® (alemtuzumab), Lymphocide® (eprtuzumab), Avastin® (bevacizumab), and Tarceva® (erlotinib), and the like. By way of example and not wishing to be bound by theory, treatment with an anti-cancer antibody or an anti-cancer antibody conjugated to a toxin can lead to cancer cell death (*e.g.,* tumor cells) which would potentiate an immune response mediated by CTLA-4, PD-1, PD-L1 or LAG-3. As an example, a treatment of a hyperproliferative disease (*e.g.,* a cancer tumor) can include an anti-cancer antibody in combination with anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibodies, concurrently or sequentially or any combination thereof, which can potentiate an anti-tumor immune responses by the host.

[0182] Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of proteins, which are expressed by

the tumors and which are immunosuppressive. These include, among others, TGF-β (Kehrl et al. (1986) J. Exp. Med. 163: 1037-1050), IL-10 (Howard & O'Garra (1992) Immunology Today 13: 198-200), and Fas ligand (Hahne et al. (1996) Science 274: 1363-1365). In another example, antibodies to each of these entities can be further combined with an anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L 1 antibody combination to counteract the effects of immunosuppressive agents and favor anti-tumor immune responses by the host.

[0183] Other antibodies that can be used to activate host immune responsiveness can be further used in combination with an anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibody combination. These include molecules on the surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 antibodies (Ridge *et al., supra*) can be used in conjunction with an anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 combination (Ito *et al., supra*). Other activating antibodies to T cell costimulatory molecules Weinberg *et al., supra,* Melero *et al. supra,* Hutloff *et al., supra*) may also provide for increased levels of T cell activation.

[0184] As discussed above, bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. A combined LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade can be used to increase the effectiveness of the donor engrafted tumor specific T cells.

[0185] Several experimental treatment protocols involve *ex vivo* activation and expansion of antigen specific T cells and adoptive transfer of these cells into recipients in order to antigen-specific T cells against tumor (Greenberg & Riddell, *supra*). These methods can also be used to activate T cell responses to infectious agents such as CMV. *Ex vivo* activation in the presence of anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibodies can be expected to increase the frequency and activity of the adoptively transferred T cells.

[0186] Further, a method for altering an adverse event associated with treatment of a hyperproliferative disease (*e.g.*, cancer) with an immunostimulatory agent, comprising administering an anti-LAG-3 antibody and a subtherapeutic dose of anti-CTLA-4 and/or anti-PD-1and/or anti-PD-L1 antibody to a subject, is described herein. For example, the methods described herein provide for a method of reducing the incidence of immunostimulatory therapeutic antibody-induced colitis or diarrhea by administering a non-absorbable steroid to the patient. Because any patient who will receive an immunostimulatory therapeutic antibody is at risk for developing colitis or diarrhea induced by such an antibody, this entire patient population is suitable for therapy according to the present invention. Although steroids have been administered to treat inflammatory bowel disease (IBD) and prevent exacerbations of IBD, they have not been used to prevent (decrease the incidence of) IBD in patients who have not been diagnosed with IBD. The significant side effects associated with steroids, even non-absorbable steroids, have discouraged prophylactic use.

[0187] Further, a combination LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade (*i.e.*, immunostimulatory therapeutic antibodies anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1

antibodies and/or anti-PD-L1 antibodies) can be further combined with the use of any non-absorbable steroid. As used herein, a "non-absorbable steroid" is a glucocorticoid that exhibits extensive first pass metabolism such that, following metabolism in the liver, the bioavailability of the steroid is low, *i.e.,* less than about 20%. The non-absorbable steroid may be budesonide. Budesonide is a locally-acting glucocorticosteroid, which is extensively metabolized, primarily by the liver, following oral administration. ENTOCORT EC® (Astra-Zeneca) is a pH- and time-dependent oral formulation of budesonide developed to optimize drug delivery to the ileum and throughout the colon. ENTOCORT EC® is approved in the U.S. for the treatment of mild to moderate Crohn's disease involving the ileum and/or ascending colon. The usual oral dosage of ENTOCORT EC® for the treatment of Crohn's disease is 6 to 9 mg/day. ENTOCORT EC® is released in the intestines before being absorbed and retained in the gut mucosa. Once it passes through the gut mucosa target tissue, ENTOCORT EC® is extensively metabolized by the cytochrome P450 system in the liver to metabolites with negligible glucocorticoid activity. Therefore, the bioavailability is low (about 10%). The low bioavailability of budesonide results in an improved therapeutic ratio compared to other glucocorticoids with less extensive first-pass metabolism. Budesonide results in fewer adverse effects, including less hypothalamic-pituitary suppression, than systemically-acting corticosteroids. However, chronic administration of ENTOCORT EC® can result in systemic glucocorticoid effects such as hypercorticism and adrenal suppression. *See* PDR 58th ed. 2004; 608-610.

[0188] Still further, a combination LAG-3 and CTLA-4 and/or PD-1 and/or PD-L1 blockade (*i.e.*, immunostimulatory therapeutic antibodies anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibodies) in conjunction with a non-absorbable steroid can be further combined with a salicylate. Salicylates include 5-ASA agents such as, for example: sulfasalazine (AZULFIDINE®, Pharmacia & UpJohn); olsalazine (DIPENTUM®, Pharmacia & UpJohn); balsalazide (COLAZAL®, Salix Pharmaceuticals, Inc.); and mesalamine (ASACOL®, Procter & Gamble Pharmaceuticals; PENTASA®, Shire US; CANASA®, Axcan Scandipharm, Inc.; ROWASA®, Solvay).

[0189] In accordance with the methods described herein, a salicylate administered in combination with anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibodies and a non-absorbable steroid can include any overlapping or sequential administration of the salicylate and the non-absorbable steroid for the purpose of decreasing the incidence of colitis induced by the immunostimulatory antibodies. Thus, for example, methods for reducing the incidence of colitis induced by the immunostimulatory antibodies encompass administering a salicylate and a non-absorbable concurrently or sequentially (*e.g.*, a salicylate is administered 6 hours after a non-absorbable steroid), or any combination thereof. Further, a salicylate and a non-absorbable steroid can be administered by the same route (*e.g.*, both are administered orally) or by different routes (*e.g.*, a salicylate is administered orally and a non-absorbable steroid is administered rectally), which may differ from the route(s) used to administer the anti-LAG-3 and anti-CTLA-4 and/or anti-PD-1 and/or anti-PD-L1 antibodies.

[0190] The present disclosure is further illustrated by the following examples, which should not be construed as further limiting.

Examples

Example 1: Design of Variants of LAG3.1 (Antibody 25F7)

[0191] Antibody variants of the previously described anti-LAG-3 antibody, 25F7, referred to herein as LAG3.1, were created by first analyzing the amino acid sequence of the antibody for potential sites of degradation. Expression of site-directed mutagenesis of LAG3.1 V_H region was performed using QuikChange II XL® Site-Directed Mutagenesis Kit (Agilent Technologies). The altered V_H regions were then subcloned into UCOE® (EMD Millipore) vectors that contain the human IgG4-S228P constant region. The various heavy chain vectors were each co-transfected with a vector expressing the LAG3.1 kappa chain into CHO-S cells, and stable pools were selected for expression

[0192] Five potential deamidation motifs were identified within the variable region heavy chain CDR2. These sites were located at positions 52, 54, 56, 58, and 60 of the heavy chain variable region of LAG3.1 (SEQ ID NO: 2) (see Figure 1A). In particular, deamidation of the "NG" sequence within the VH CDR2 (SEQ ID NO: 6) was observed under all conditions, as well as further isomerization of the sequence. Deamidation of the starting material was about 10%. Further, it was found that this "NG" sequence did not correspond to a germline sequence (see Figure 3). However, the consensus germline sequence was a potential glycosylation site and, therefore, was not included among the antibody variants.

[0193] Four variants (referred to herein as LAG3.5, LAG3.6, LAG3.7, and LAG3.8) were designed which addressed two of the potential deamidation motifs (positions 54 and 56), as shown in Figure 3. These variants were subjected to a matrix of conditions as summarized in Table 1 below and the following characteristics were analyzed: (a) chemical and thermal stabilities (physical stability); (b) size exclusion chromatography (aggregation); (c) Isoelectric Focusing gel (IEF) (charge heterogeneity); (d) activity by Biacore analysis (binding and functional activity); and (e) peptide mapping by mass-spectrometry (chemical modifications / molecular stability).

Table 1

Buffer	Acetate (100nM NaCl, 3% w/v mannitol, 0.03% Tween-20)	Citrate (100nM NaCl, 3% w/v mannitol, 0.03% Tween-20)
pH	5.5, 6.0, 6.5, 7.0	5.5, 6.0, 6.5, 7.0
Temperature	4°C and 37°C	4°C and 37°C
Time	0, 4, 8, 12 weeks	0, 4, 8, 12 weeks

Example 2: Characterization of LAG-3 Variants

1. Activated human $CD4^+$ T Cell Binding

[0194] To test the ability of the antibody variants to bind to native human LAG-3 on the surface of activated human T cells, normal healthy donor peripheral blood mononuclear cells were stimulated in 15 cm tissue culture plates at a density of 2x10e6 cells/mL, with a combination of anti-CD3 (eBioscience, Cat #16-0037-85) and anti-CD28 (BD Bioscience, Cat # 555725) antibodies present in solution at 5 µg/mL and 3 µg/mL, respectively. Following three days of stimulation cells were harvested, washed 1X with 1x PFAE buffer (1x PBS + 2% FBS, 0.02% sodium azide, 2mM Na EDTA), and resuspended in 1x PFAE buffer for staining.

[0195] For the binding reaction, the LAG3.1 variants were serially diluted with cold 1x PFAE buffer, then 50 µl of diluted antibody solution was mixed with 50 µl of Fitc-labeled anti-human CD4 (BD Bioscience, Cat # 555346) diluted 1:16 in 1x PFAE buffer. For the binding reaction, 100 µl of this diluted antibody mixture was added to 2×10^5 cells and the mixture was incubated on at 4°C for 30 minutes. The cells were then washed two times with 1x PFAE buffer. A 1:200 dilution of PE-labeled goat anti-human Fcy-specific antibody (Jackson ImmunoResearch, Cat. # 109-116-170) was added and the mixture was incubated for 30 minutes at 4°C, followed by washing twice with cold 1x PFAE buffer. After the final wash, 150 µl of cold 1x PFAE was added to each solution and analysis of antibody binding was carried out by flow cytometry using a FACSCanto flow cytometer (BD Bioscience).

[0196] The results of the flow cytometry analysis are summarized in Figure 4A which is a graph showing the EC_{50} for antibody binding to activated human CD4+ T cells. Figure 4B is a graph showing antibody binding to soluble human LAG-3/Fc antigen by BIACORE. As shown, the binding affinities of LAG3.5 and LAG3.8 are slightly lower, compared to LAG3.1, while their off-rate constants are slightly higher compared to LAG3.1.

2. Physical Stability

[0197] Thermal stability and thermal denaturation of the variants was tested using Microcal VP-DSC. Specifically, each variant was diluted into PBS (Mediatech cat # 21-040-CV lot # 21040139). The final concentration of sample was 250 µg/mL after dilution into PBS. The sample was scanned to 74°C, cooled to 25°C, and reheated to 74°C. PBS buffer was used as a blank control. Data was fit to a Non-2-state model and curve fitting performed by Origin software.

[0198] As summarized in Table 2 and shown in Figure 5, LAG3.5 had a higher melting temperature TM2 than LAG3.1, indicating greater overall stability.

Table 2

MAb	Tm1 (°C) Corresponds to CH2 and/or Fab domains	Tm2 (°C) Corresponds to CH3 and/or Fab domains
LAG3.1	70.7	75.7
LAG3.5	70.5	76.3
LAG3.6	67.8	70.8
LAG3.7	69.4	73.5
LAG3.8	70.3	75.4

[0199] Antibody refolding following denaturation is an inverse measure of long-term aggregation potential. Accordingly, the LAG-3 variants also were tested and compared in terms of thermal reversability. Specifically, the antibodies were heated to 74° C and cooled to room temperature before heated back to 74° C. The ratio of area under the curve of the second to first thermograms provides the estimate of thermal reversibility, which is a direct measure of conformational reversibility.

[0200] As summarized in Table 3 and shown in Figure 6, LAG3.5 had substantially higher thermal reversibility than all other variants. Notably, the percent reversibility for LAG3.5 (47%) was more than double that of LAG3.1 (20%). The thermal reversibility is strongly correlated to the long-term aggregation potential. Lower reversibility corresponds to higher potential aggregation. Based on this observation, LAG3.1 would potentially exhibit substantially higher aggregation over time, compared to LAG3.5. Similarly, all other variants could potentially exhibit substantially higher aggregation over time compared to LAG3.5.

Table 3

MAb	Thermal reversibility (%)
LAG3.1	20
LAG3.5	47
LAG3.6	0
LAG3.7	11
LAG3.8	26

3. Aggregation

[0201] The variants also were tested for stability as a measure of protein aggregation using standard Size Exclusion HPLC (SEC-HPLC) according the following protocol: antibody test samples were diluted to 1.0 mg/ml with phosphate buffered saline (PBS) and 10 uL was

applied to an HPLC (Waters, model 2795). Separation was accomplished on a gel filtration column (TOSOH Bioscience, TSKgel G3000 SWxl, 7.8mm x 300mm, product #08541) using a mobile phase of 0.1M sodium phosphate, 0.15M sodium chloride, 0.1M sodium sulfate, pH 7.2. The analyte was detected by monitoring UV absorbance at 280nm, and the antibody peak area percent composition was determined using Empower software. As shown in Table 4, LAG3.5 exhibited substantially reduced aggregation compared to LAG3.1.

Table 4

Sample	IgG Monomer (% peak area)	IgG Aggregate (% peak area)
LAG3.1	90	10
LAG3.5	96	4
LAG3.6	96	4
LAG3.7	95	5
LAG3.8	95	5

Example 3: Variant Selection

[0202] Based on the studies described above, antibody variant LAG3.5 was selected for further analysis, in view of its significantly improved physical and chemical stability compared to its unmodified form (LAG3.1), particularly its high capacity for conformational refolding (thermal reversibility). This analysis included a two-step approach of (a) accelerated stress, (b) followed by 12-week real-time stability evaluation. Specifically, LAG3.5 was incubated at 1.0 mg/ml in pH 8.0, 50 mM Ammonium Bicarbonate, for 5 days at 40C°. The degree of modifications after 5 days was analyzed, as well as the effects on activity and stability. The LAG3.5 variant was then subjected to real-time stability in PBS for a duration of 12 weeks and subsequently analyzed. The results of these studies are described below.

1. Antigen Binding

[0203] As shown in Figure 7 (and Table 5), no change in antigen binding was observed after 5 days. As also shown in Figures 10 A and B, LAG3.5 exhibited no change in antigen binding or physical stability after 12 weeks. In particular, LAG3.5 maintains higher affinity than LAG3.8 over the entire 12 week period at both 4°C and 40°C.

Table 5

Clone ID	Antigen	$K_D \times 10^{-9}$ (M)	$k_{on} \times 10^4$ (1/Ms)	$K_{off} \times 10^{-4}$ (1/s)
Lag3.1	PBS	0.21	166	3.44
	pH8	0.20	184	3.61
Lag3.5	PBS	0.25	130	3.22

Clone ID	Antigen	K_D x 10^{-9} (M)	k_{on} x 10^4 (1/Ms)	K_{off} x 10^{-4} (1/s)
	pH8	0.20	148	2.98
Lag3.8	PBS	0.25	147	3.68
	pH8	0.25	162	4.02

2. Chemical Modifications / Molecular Stability

[0204] Peptide mapping by mass spectrometry was used to analyze the chemical / molecular stability of LAG3.5 compared to LAG3.1. Specifically, purified antibody was reduced, alkylated, dialyzed, and digested with trypsin (Promega Cat. V5111) and GluC (Roche Cat. 11047817001). Digests were analyzed by nano-LC MSMS mass spectrometry (Thermo Fisher LTQ Orbitrap).

[0205] As shown in Figure 8, LAG3.1 showed increased heterogeneity in V_H compared to LAG3.5 when subjected to accelerated stability at higher pH, which deamidates asparagine residues (step 1). Change in mass due to isomerization could not be detected under the current experimental conditions. The percentage change is expressed as a ratio of all changes combined to the parental peak.

[0206] In addition, as shown in Figure 11, LAG3.1 showed increased heterogeneity in V_H compared to LAG3.5 when subjected to prolonged real-time stability of 12 weeks, at both 4°C and 40°C (step 2).

3. Physical Stability

[0207] Thermal reversibility was measured in PBS and at pH 8.0. Under both conditions, LAG3.5 again exhibited approximately double the level of refolding compared to LAG3.1. Specifically, as shown in Tables 6-8, LAG3.5 exhibited 43% refolding compared to 18% for LAG3.1 in PBS. LAG3.5 also exhibited 48% refolding compared to 29% refolding for LAG3.1 at pH 8.0.

Table 6 - DSC:melting

MAb	Condition	Tm1	Tm2
Lag3.1	PBS	70.7	75.7
Lag3.1	pH8	70.4	75.6
Lag3.5	PBS	70.8	76.4
Lag3.5	pH8	70.5	76.3

Table 7 - Fluorolog-2:unfolding

Mab/mutants	Midpoint (M)	Aggregation (M)
Lag3.1 PBS	1.99	-
Lag3.1 pH8	2.08	-
Lag3.5 PBS	1.86	-
Lag3.5 pH8	2.00	-

Table 8: DSC:refolding

MAb	%reversibility PBS	%reversibility pH8
Lag3.1	18	29
Lag3.5	43	48

4. Charge Heterogeneity

[0208] To assess charge heterogeneity, the variants were analyzed using isoelectrofocusing (IEF) with standard markers of pI 5.5 and pI 10.0 compared to LAG3.1. Briefly, antibody solutions were applied onto a 1 mm thick IEF pI 3-7 pre-made gel (Invitrogen, Cat# EC6648BOX) along with a pI 3-10 markers (SERVA, Cat# 39212). Electrophoresis was carried out using IEF 3-7 Cathode buffer (Invitrogen, Cat# LC5370) and IEF Anode buffer (Invitrogen, Cat# LC5300) and applying electrical current in the order of 100 V constant for 1 hr, 200 V constant for 1 hr, and 500 V constant for 30 min. The IEF gels were stained with Coomassie blue to detect the protein bands and destained with methanol-acetic acid solution. IEF gels were then analyzed by ImageQuant TL software. Based on this analysis (data not shown), LAG3.5 exhibited significantly less heterogeneity compared to LAG3.1.

5. HIC-HPLC

[0209] To assess solubility, the variants were analyzed using standard Hydrophobic Interaction Chromatography (HIC-HPLC) according to the following protocol: 50 uL of 2M ammonium sulfate was added to 50 uL of antibody test sample at 1 mg/ml. 80 uL of the test sample was then applied to an HPLC (Waters, model 2795) connected in-line to an HIC column (TOSOH Bioscience, Ether-5PW TSK-gel, 7.5mm x 75mm, product #07573). The sample was eluted at a flow rate of 1.0 ml/min with a gradient of 100% buffer A (2M ammonium sulfate, 0.1M sodium phosphate, pH 7.0) to 100% buffer B (0.1M sodium phosphate, pH 7.0) over 50 minutes. The antibody was detected by monitoring UV absorbance at 280nm and data was analyzed using Empower software. As shown in Figure 9, the hydrophilicity of LAG3.5 exhibited solubility at high concentrations of ammonium sulfate.

Example 4: Reversal of T-Cell Mediated Immune Response Inhibition

[0210] The activity of LAG3.5 was determined by means of a functional assay that utilized an antigen-specific mouse T cell hybridoma (3A9). Hybridoma 3A9 expresses a T cell receptor specific for a peptide from hen egg lysozyme (HEL48-62) and secretes IL-2 when co-cultured with peptide-pulsed, MHC-matched, antigen presenting cells (LK35.2). Since huLAG-3-Fc is capable of binding to MHC Class II-positive mouse B cell lines, expression of huLAG-3 in the 3A9 line could exert an inhibitory effect through engagement with Class II on the murine presenting line. A comparison of the peptide response profile of the 3A9 parent with that of the human LAG-3-transduced 3A9 cells co-cultured with MHC-matched antigen presenting cells demonstrated that the expression of human LAG-3 inhibited peptide responsiveness compared to control 3A9 cells. This inhibition was reversed by LAG-3 blockade using LAG3.5. Therefore, blockade of LAG-3-mediated inhibition was demonstrated for LAG3.5.

Example 5: T-Cell Activation by LAG3.5

[0211] The functional activity of LAG3.5 on primary T cells was assessed using human PBMC cultures stimulated by the superantigen SEB. Total PBMC were isolated from the blood of eighteen human donors and stimulated for 72 hours in either of two assay formats: (i) a fixed amount of antibody (20 µg/mL) and serial dilutions of SEB, or (ii) a fixed amount of SEB (85 ng/mL) and serial dilutions of antibody. Secreted IL-2, as a measure of T cell activity, was monitored by ELISA. Antibody anti-PD-1 antibody and Ipilimumab were used as positive controls and the activity of LAG3.5 in combination with anti-PD-1 or anti-CTLA-4 was also evaluated for a subset of donors.

[0212] Enhanced IL-2 secretion was observed over a range of SEB concentrations from fifteen of the eighteen donors treated with LAG3.5 alone, compared to isotype control antibody treatment. In most instances the stimulation was less than that observed for treatment with anti-PD-1 or Ipilimumab. With respect to LAG3.5, the results of the two assay formats (described above) were in agreement with one another. Moreover, in 5 of 6 donors tested, combining LAG3.5 with anti-PD-1 or Ipilimumab resulted in higher levels of stimulation than observed for isotype control antibody combined with anti-PD-1 or Ipilimumab. These data revealed that LAG3.5 can function in normal human T cell assays and can further activate responses mediated by inhibition of PD-1 and CTLA-4 function.

SUMMARY OF SEQUENCE LISTING

[0213]

SEQ ID NO:	DESCRIPTION	SEQUENCE
1	V_H n.a. 25F7 (LAG3.1)	

>1408_LAG-3_403_25F7.1_VH1_NT

CAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGCCTTCGGAGACC
CTGTCCCTCACCTGCGCTGTCTATGGTGGGTCCTTCAGTGATTACTACTGGAA
CTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAA
TCATAATGGAAACACCAACTCCAACCCGTCCCTCAAGAGTCGAGTCACCCTA
TCACTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGGTCTGTGACCG
CCGCGGACACGGCTGTGTATTACTGTGCGTTTGGATATAGTGACTACGAGTA
CAACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

2	V_H a.a. 25F7	

>1408_LAG-3_403_25F7.1_VH1_AA
QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPGKGLEWIGEINH
NGNTNSNPSLKSRVTLSLDTSKNQFSLKLRSVTAADTAVYYCAFGYSDYEYNW
FDPWGQGTLVTVSS

3	V_K n.a. 25F7	

>1408_LAG-3_403_25F7.1_VK1_NT
GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAA
GAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTAGCAGCTACTTAGCCTG
GTACCAACAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCC
AACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
GACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATT
ACTGTCAGCAGCGTAGCAACTGGCCTCTCACTTTTGGCCAGGGGACCAACCT
GGAGATCAAA

4	V_K a.a. 25F7	

>1408_LAG-3_403_25F7.1_VK1_AA
EIVLTQSPATLSLSPGERATLSCRASQSISSYLAWYQQKPGQAPRLLIYDASNRAT
GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGQGTNLEIK

5	V_H CDR1 a.a. 25F7	DYYWN
6	V_H CDR2 a.a. 25F7	EINHNGNTNSNPSLKS
7	V_H CDR3 a.a. 25F7	GYSDYEYNWFDP
8	V_K CDR1 a.a. 25F7	RASQSISSYLA
9	V_K CDR2 a.a. 25F7	DASNRAT
10	V_K CDR3 a.a. 25F7	QQRSNWPLT
11	V_H n.a. LAG3.5	

V_H n.a. LAG3.5
caggtgcagctacagcagtggggcgcaggactgttgaagccttcggagaccctgtccctcacctgcgctgtctatggtgggtc
cttcagtgattactactggaactggatccgccagccccagggaaggggctggagtggattggggaaatcaatcatcgtggaa
gcaccaactccaacccgtccctcaagagtcgagtcaccctatcactagacacgtccaagaaccagttctccctgaagctgaggt
ctgtgaccgccgcggacacggctgtgtattactgtgcgtttggatatagtgactacgagtacaactggttcgacccctggggcc
agggaaccctggtcaccgtctcctca

12	V_H a.a. LAG3.5	

V_H a.a. LAG3.5

QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPGKGLEWIGE
INHRGSTNSNPSLKSRVTLSLDTSKNQFSLKLRSVTAADTAVYYCAFGYS
DYEYNWFDPWGQGTLVTVSS

13	V_K n.a. LAG3.5	

V_K n.a. LAG3.5

gaaattgtgttgacacagtctccagccaccctgtctttgtctccaggggaaagagccaccctctcctgcagggccagtcagagt

attagcagctacttagcctggtaccaacagaaacctggccaggctcccaggctcctcatctatgatgcatccaacagggccact

ggcatcccagccaggttcagtggcagtgggtctgggacagacttcactctcaccatcagcagcctagagcctgaagattttgca

gtttattactgtcagcagcgtagcaactggcctctcacttttggccaggggaccaacctggagatcaaa

14	V_K a.a. LAG3.5	

V_K a.a. LAG3.5

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ASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGQ
GTNLEIK

15	V_H CDR1 a.a. LAG3.5	**DYYWN**
16	V_H CDR2 a.a. LAG3.5	**EINHRGSTNSNPSLKS**
17	V_H CDR3 a.a. LAG3.5	GYSDYEYNWFDP
18	V_K CDR1 a.a. LAG3.5	RASQSISSYLA
19	V_K CDR2 a.a. LAG3.5	DASNRAT
20	V_K CDR3 a.a. LAG3.5	**QQRSNWPLT**
21	LAG-3 epitope	PGHPLAPG
22	LAG-3 epitope	HPAAPSSW
23	LAG-3 epitope	PAAPSSWG
24	V_H CDR2 a.a. LAG3.6	**EIIHSGSTNSNPSLKS**
25	V_H CDR2 a.a. LAG3.7	**EINHGGGTNSNPSLKS**
26	V_H CDR2 a.a. LAG3.8	**EINHIGNTNSNPSLKS**
27	V_H CDR2 a.a.HUMAN GERMLINE	GEINHSGSTNY
28		$(Gly_4\text{-}Ser)_3$
29	Human LAG-3 a.a.	

human LAG-3 a.a. sequence

MWEAQFLGLLFLQPLWVAPVKPLQPGAEVPVVWAQEGAPAQLPCSPTIPLQDL
SLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPHPAAPSSWGPRPRRYTVLSVGP
GGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPARRADAGEYRAAVHLRDRALS
CRLRLRLGQASMTASPPGSLRASDWVILNCSFSRPDRPASVHWFRNRGQGRVPV
RESPHHHLAESFLFLPQVSPMDSGPWGCILTYRDGFNVSIMYNLTVLGLEPPTPL
TVYAGAGSRVGLPCRLPAGVGTRSFLTAKWTPPGGGPDLLVTGDNGDFTLRLE

DVSQAQAGTYTCHIHLQEQQLNATVTLAIITVTPKSFGSPGSLGKLLCEVTPVSG
QERFVWSSLDTPSQRSFSGPWLEAQEAQLLSQPWQCQLYQGERLLGAAVYFTE
LSSPGAQRSGRAPGALPAGHLLLFLTLGVLSLLLLVTGAFGFHLWRRQWRPRRF
SALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPEQL*

30	V_H CDR2 a.a. LAG3.2	**VIWYDGSNKYYADSVKG**
31	V_HLAG3.1 n.a.	

LAG3.1HC

CAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGCCTTCGGAGACC
CTGTCCCTCACCTGCGCTGTCTATGGTGGGTCCTTCAGTGATTACTACTGGAA
CTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAA
TCATAATGGAAACACCAACTCCAACCCGTCCCTCAAGAGTCGAGTCACCCTA
TCACTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGGTCTGTGACCG
CCGCGGACACGGCTGTGTATTACTGTGCGTTTGGATATAGTGACTACGAGTA
CAACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGCT
AGCACCAAGGGCCCATCCGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCT
CCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACC
GGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTC
CCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCG
TGCCCTCCAGCAGCTTGGGCACGAAGACCTACACCTGCAACGTAGATCACA
AGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGTCCAAATATGGTCCCC

CATGCCCACCATGCCCAGCACCTGAGTTCCTGGGGGGACCATCAGTCTTCCT
GTTCCCCCCAAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTC
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TGGTACGTGGATGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAG
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AGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCC
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AGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGT
GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCC
CGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAGGCTAACCGTGGAC
AAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATGCATGAG
GCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAAT
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32	V_HLAG3.1 a.a.	

TRANSLATION\OF\LAG3.1HC

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FDPWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKV
DKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQE
DPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
EALHNHYTQKSLSLSLGK*

33	V_LLAG3.1 n.a.	

LAG3.1LC

GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAA
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AACAGGGCCACTGGCATCCCAGCCAGGTTCAGTGGCAGTGGGTCTGGGACA
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ACTGTCAGCAGCGTAGCAACTGGCCTCTCACTTTTGGCCAGGGGACCAACCT
GGAGATCAAACGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCT
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TCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAAT
CGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCT
ACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACA
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GAGCTTCAACAGGGGAGAGTGTTAG

34	V_LLAG3.1 a.a.	

TRANSLATION\OF\LAG3.1LC

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GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGQGTNLEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*

35	V_HLAG3.5 a.a.	

LAG3.5 heavy chain sequence - complete

QVQLQQWGAGLLKPSETLSLTCAVYGGSFSDYYWNWIRQPPGKGLEWIGE
INHRGSTNSNPSLKSRVTLSLDTSKNQFSLKLRSVTAADTAVYYCAFGYS
DYEYNWFDPWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKT
YTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDT
LMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYT
LPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK*

36	V_HLAG3.5 n.a.	

LAG3.5 heavy chain sequence - complete

caggtgcagctacagcagtggggcgcaggactgttgaagccttcggagaccctgtccctcacctgcgctgtctatggtgggtc
cttcagtgattactactggaactggatccgccagccccccagggaaggggctggagtggattggggaaatcaatcatcgtggaa
gcaccaactccaacccgtccctcaagagtcgagtcaccctatcactagacacgtccaagaaccagttctccctgaagctgaggt
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tgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctaccccagcgacatcgccgtggagtgggagagcaatgg
gcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctccttcttcctctacagcaggctaaccgtgg
acaagagcaggtggcaggaggggaatgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacacagaagag
cctctccctgtctctgggtaaatga

37	V_LLAG3.5 a.a.	

LAG3.5 kappa chain sequence - Complete

EIVLTQSPATLSLSPGERATLSCRASQSISSYLAWYQQKPGQAPRLLIYD
ASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGQ
GTNLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGEC*

38	V_LLAG3.5 n.a.	

LAG3.5 - kappa chain sequence - Complete

gaaattgtgttgacacagtctccagccaccctgtctttgtctccaggggaaagagccaccctctcctgcagggccagtcagagt
attagcagctacttagcctggtaccaacagaaacctggccaggctcccaggctcctcatctatgatgcatccaacagggccact
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aaggacagcacctacagcctcagcagcaccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaa
gtcacccatcagggcctgagctcgcccgtcacaaagagcttcaacaggggagagtgttag

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OPTIMERING AF ANTISTOFFER, DER BINDER LYMFOCYTAKTIVERINGSGEN-3 (LAG-3), OG ANVENDELSER DERAF

PATENTKRAV

1. Enkelt sammensætning, der omfatter:

(a) et monoklonalt antistof eller en antigenbindende del deraf, der binder humant lymfocytaktiveringsgen-3 (LAG-3),

(b) et anti-PD-1-antistof eller en antigenbindende del deraf, og

(c) en farmaceutisk acceptabel bærer;

hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, omfatter tungekæde-CDR1-, CDR2- og CDR3-områder omfattende aminosyresekvenserne ifølge henholdsvis SEQ ID NO: 15, 16 og 17, og letkæde-CDR1-, CDR2- og CDR3-områder, der omfatter aminosyresekvenserne ifølge henholdsvis SEQ ID NO: 18, 19 og 20.

2. Enkelt sammensætning ifølge krav 1, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, omfatter variable tungkæde- og letkædeområder omfattende aminosyresekvenserne ifølge henholdsvis SEQ ID NO: 12 og 14.

3. Enkelt sammensætning ifølge krav 1 eller 2, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, har én eller en kombination af følgende egenskaber:

(a) binding til abe-LAG-3;

(b) manglende binding til muse-LAG-3;

(c) hæmmer binding af LAG-3 til større histokompatibilitets- (MHC) klasse II-molekyler; eller

(d) stimulerer et immunrespons.

4. Enkelt sammensætning ifølge et hvilket som helst af krav 1-3, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, stimulerer interleukin-2 (IL-2)-produktion i et antigenspecifikt T-cellerespons og/eller stimulerer et antitumor-immunrespons.

5. Enkelt sammensætning ifølge et hvilket som helst af krav 1-4, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, er et fuldlængde antistof og/eller anti-PD-1-antistoffet eller den antigenbindende del deraf er et fuldlængde antistof.

6. Enkelt sammensætning ifølge et hvilket som helst af krav 1-5, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, er en IgG1-, IgG2- eller IgG4-isotype og/eller anti-PD-1-antistoffet eller den antigenbindende del deraf er en IgG1-, IgG2- eller IgG4-isotype.

7. Enkelt sammensætning ifølge et hvilket som helst af krav 1-6, hvor det monoklonale antistof, der binder til human LAG-3, er et fuldlængde humant IgG4-antistof, der binder human LAG-3 med et K_D på $0{,}27 \times 10^{-9}$ M eller mindre bestemt ved overfladeplasmonresonans.

8. Enkelt sammensætning ifølge et hvilket som helst af krav 1-7, hvor det monoklonale antistof, der binder human LAG-3, omfatter tung- og letkæder omfattende aminosyresekvenserne ifølge henholdsvis SEQ ID NO: 35 og 37.

9. Enkelt sammensætning ifølge et hvilket som helst af krav 1-4, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, er et antistoffragment eller et enkeltkædeantistof og/eller anti-PD-1-antistoffet eller den antigenbindende del deraf er et antistoffragment eller et enkeltkædeantistof.

10. Enkelt sammensætning ifølge et hvilket som helst af krav 1-9, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3, er et humant, humaniseret eller kimærisk antistof, og anti-PD-1-antistoffet eller den antigenbindende del deraf er et humant, humaniseret eller kimærisk antistof.

11. Enkelt sammensætning ifølge et hvilket som helst af krav 1-10, hvor det monoklonale antistof eller den antigenbindende del deraf, der binder human LAG-3 og anti-PD-1-antistoffet eller den antigenbindende del deraf, er monoklonale antistoffer med human sekvens.

12. Enkelt sammensætning ifølge et hvilket som helst af krav 1-11, hvor anti-PD-1-antistoffet eller den antigenbindende del deraf omfatter variable tung- og letkædeområder af 5C4.

13. Enkelt sammensætning ifølge et hvilket som helst af krav 1-12, der er egnet til intravenøs administration.

14. Enkelt sammensætning ifølge et hvilket som helst af krav 1-13 til anvendelse i en fremgangsmåde til stimulering af et immunrespons hos et individ.

15. Enkelt sammensætning til anvendelse ifølge krav 14, hvor individet er et tumorbærende individ, og et immunrespons mod tumoren stimuleres.

16. Enkelt sammensætning til anvendelse ifølge krav 14, hvor immunresponset er et antigenspecifikt T-cellerespons, således at et antigenspecifikt T-cellerespons stimuleres.

17. Enkelt sammensætning til anvendelse ifølge krav 16, hvor interleukin-2-produktion ved den antigenspecifikke T-celle stimuleres.

18. Enkelt sammensætning ifølge et hvilket som helst af krav 1-13 til anvendelse i en fremgangsmåde til hæmning af tumorcellevækst hos et individ.

19. Enkelt sammensætning ifølge et hvilket som helst af krav 1-13 til anvendelse i en fremgangsmåde til behandling af cancer hos et individ.

20. Enkelt sammensætning til anvendelse ifølge krav 19, hvor canceren er melanom, metastatisk malignt melanom, renal cancer, klarcellekarcinom, prostatacancer, hormonrefraktært prostataadenokarcinom, brystcancer, coloncancer, lungecancer eller ikke-småcellet lungecancer.

21. Enkelt sammensætning til anvendelse ifølge krav 19 eller 20, hvor canceren er en refraktær eller tilbagevendende malignitet eller en metastatisk cancer.

DRAWINGS

Drawing

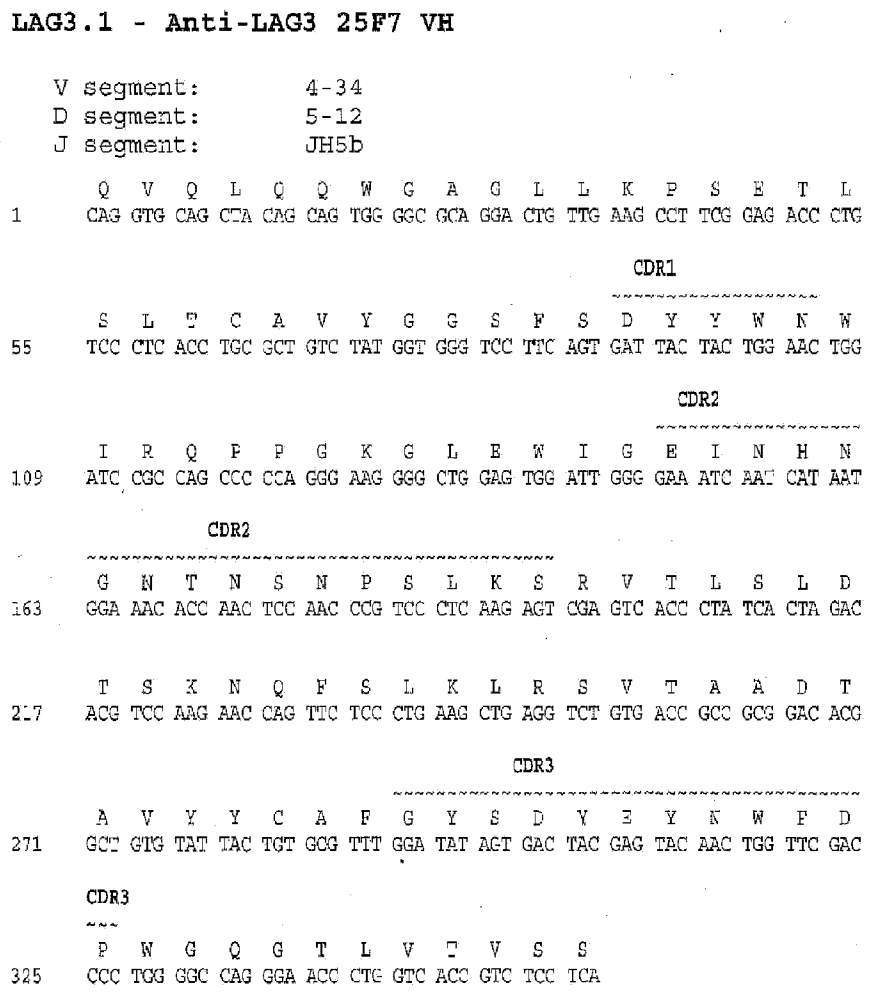


Fig. 1A

LAG3.1 - Anti-LAG3 25F7 VK

V segment: L6
J segment: JK2

```
     E  I  V  L  T  Q  S  P  A  T  L  S  L  S  P  G  E  R
1    GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

                              CDR1
                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
     A  T  L  S  C  R  A  S  Q  S  I  S  S  Y  L  A  W  Y
55   GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT ATT AGC AGC TAC TTA GCC TGG TAC

                                                    CDR2
                                                    ~~~~~~~~~~~~~~~~~~~
     Q  Q  K  P  G  Q  A  P  R  L  L  I  Y  D  A  S  N  R
109  CAA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG

     CDR2
     ~~~~~~~
     A  T  G  I  P  A  R  F  S  G  S  G  S  G  T  D  F  T
163  GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

                                                       CDR3
                                                       ~~~~~~~
     L  T  I  S  S  L  E  P  E  D  F  A  V  Y  Y  C  Q  Q
217  CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG

           CDR3
     ~~~~~~~~~~~~~~~~~~~~~~~~~~
     R  S  N  W  P  L  T  F  G  Q  G  T  N  L  E  I  K
271  CGT AGC AAC TGG CCT CTC ACT TTT GGC CAG GGG ACC AAC CTG GAG ATC AAA
```

Fig. 1B

LAG3.5 - Anti-LAG VH

Q V Q L Q Q W G A G L L K P S E T L

CDR1

S L T C A V Y G G S F S D Y Y W N W

CDR2

I R Q P P G K G L E W I G E I N H R

CDR2

G S T N S N P S L K S R V T L S L D

T S K N Q F S L K L R S V T A A D T

CDR3

A V Y Y C A F G Y S D Y E Y N W F D

CDR3

P W G Q G T L V T V S S

Fig. 2A

LAG3.5 - Anti- LAG3 VK

V segment: L6
J segment: JK2

```
     E  I  V  L  T  Q  S  P  A  T  L  S  L  S  P  G  E  R
1    GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

                              CDR1
                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
     A  T  L  S  C  R  A  S  Q  S  I  S  S  Y  L  A  W  Y
55   GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT ATT AGC AGC TAC TTA GCC TGG TAC

                                                      CDR2
                                                      ~~~~~~~~~~~~~~~~~~
     Q  Q  K  P  G  Q  A  P  R  L  L  I  Y  D  A  S  N  R
109  CAA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG

     CDR2
     ~~~~~~~
     A  T  G  I  P  A  R  F  S  G  S  G  S  G  T  D  F  T
163  GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

                                                         CDR3
                                                         ~~~~~~~
     L  T  I  S  S  L  E  P  E  D  F  A  V  Y  Y  C  Q  Q
217  CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG

          CDR3
     ~~~~~~~~~~~~~~~~~~~~~~~~~~~
     R  S  N  W  P  L  T  F  G  Q  G  T  N  L  E  I  K
271  CGT AGC AAC TGG CCT CTC ACT TTT GGC CAG GGG ACC AAC CTG GAG ATC AAA
```

Fig. 2B

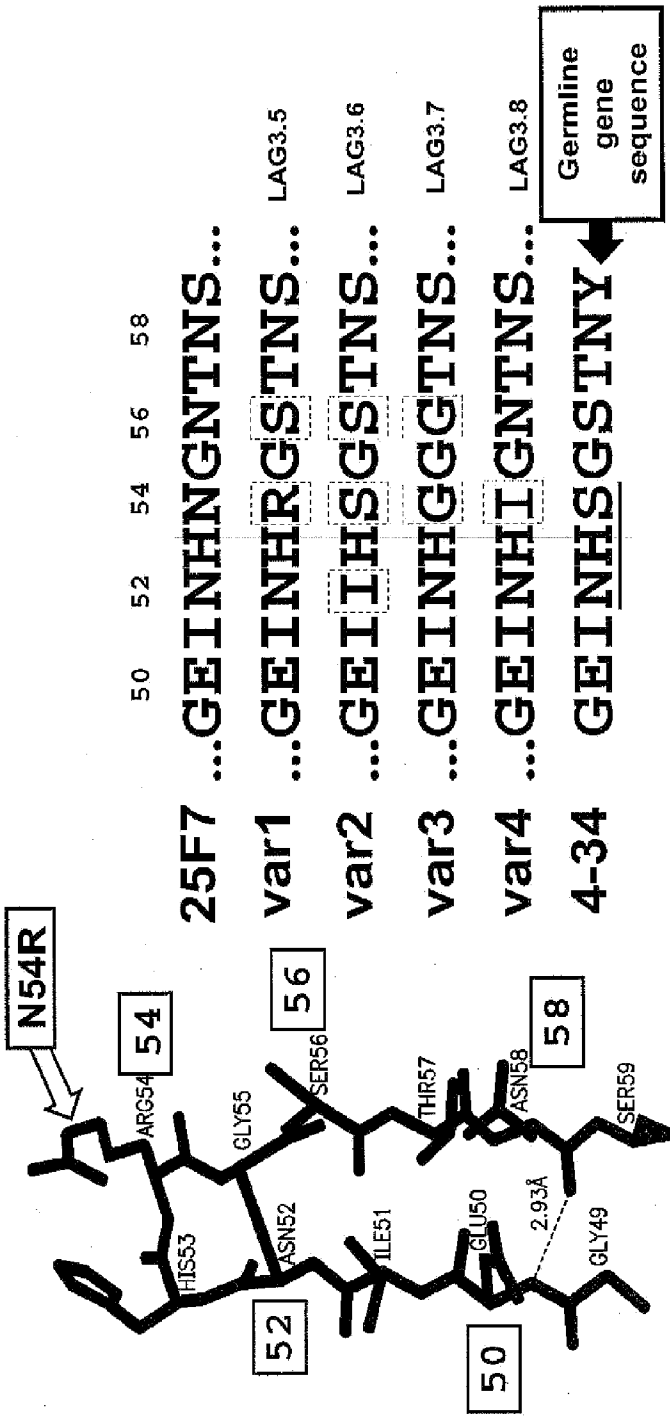
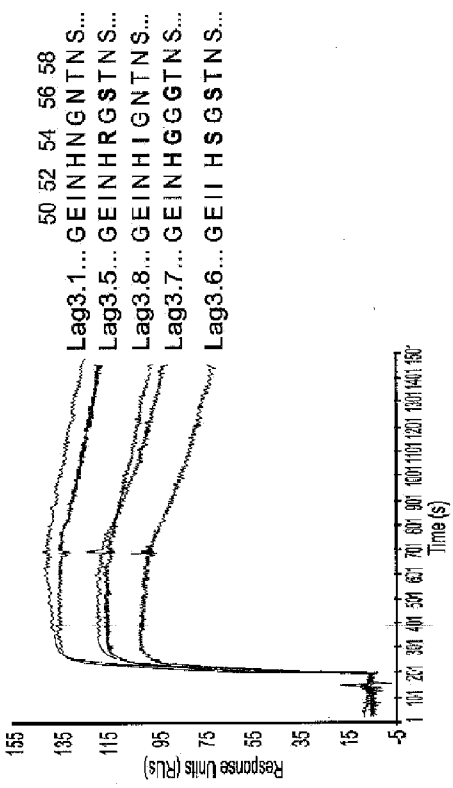

50 52 54 56 58
25F7 ...GEINHNGNTNS...
var1 ...GEINHRGSTNS... LAG3.5
var2 ...GEIIHSGSTNS... LAG3.6
var3 ...GEINHGGGTNS... LAG3.7
var4 ...GEINHIGNTNS... LAG3.8
4-34 GEINHSGSTNY
Germline gene sequence
N54R
54
56
58
52
50
ARG54
GLY55
SER56
THR57
ASN58
SER59
ASN60
PRO61
HIS53
ASN52
ILE51
GLU50
GLY49
2.93Å

Fig. 3



Clone ID	$K_D \times 10^{-9}$ (M)	$k_{on} \times 10^4$ (1/Ms)	$k_{off} \times 10^{-4}$ (1/s)
LAG3.1	0.27	102	2.79
LAG3.5	0.27	122	3.27
LAG3.6	0.66	118	7.77
LAG3.7	0.61	104	6.36
LAG3.8	0.30	130	3.36

Ag: D1D4 construct

Fig. 4B

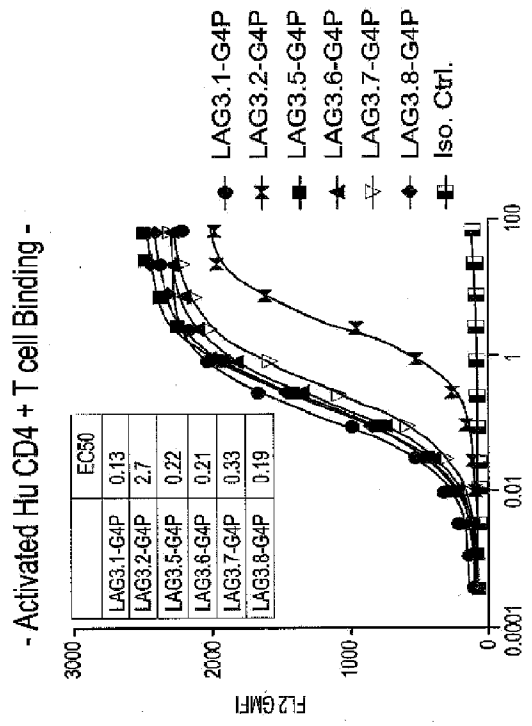


Fig. 4A

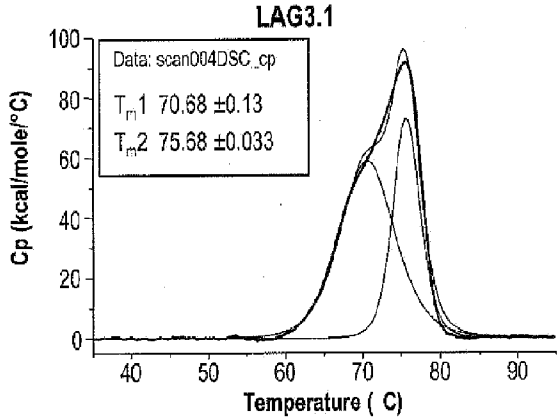


Fig. 5A

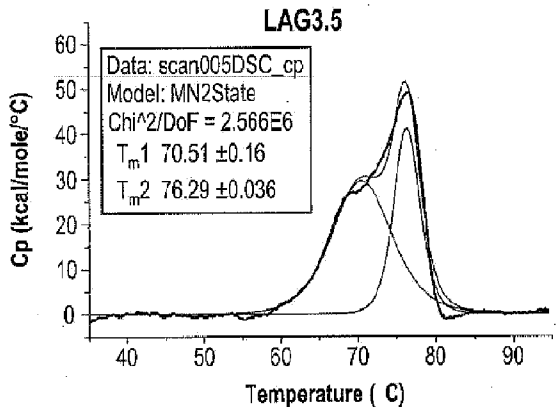


Fig. 5B

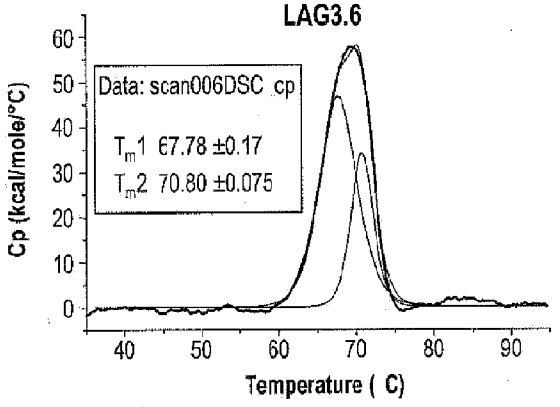


Fig. 5C

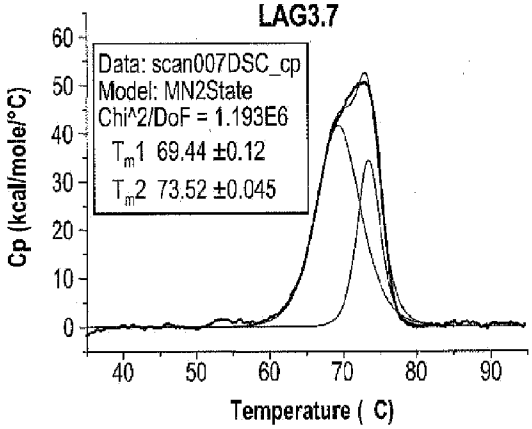

LAG3.7
Data: scan007DSC_cp
Model: MN2State
Chi^2/DoF = 1.193E6
T_m1 69.44 ±0.12
T_m2 73.52 ±0.045
Cp (kcal/mole/°C)
Temperature (C)

Fig. 5D

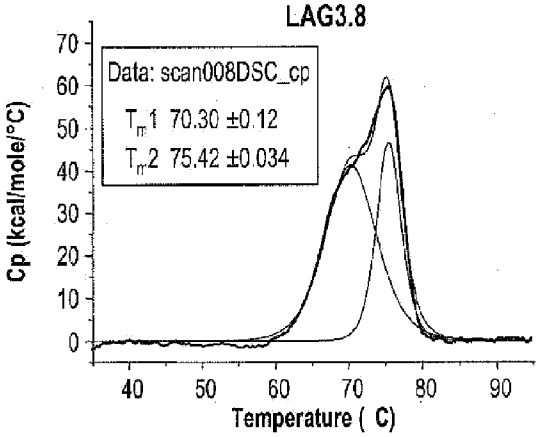

LAG3.8
Data: scan008DSC_cp
T_m1 70.30 ±0.12
T_m2 75.42 ±0.034
Cp (kcal/mole/°C)
Temperature (C)

Fig. 5E

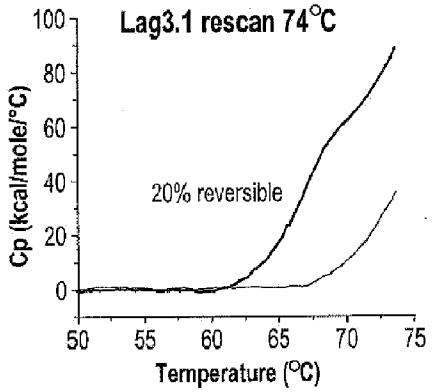


Fig. 6A

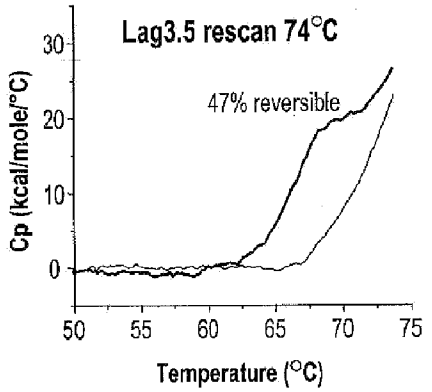


Fig. 6B

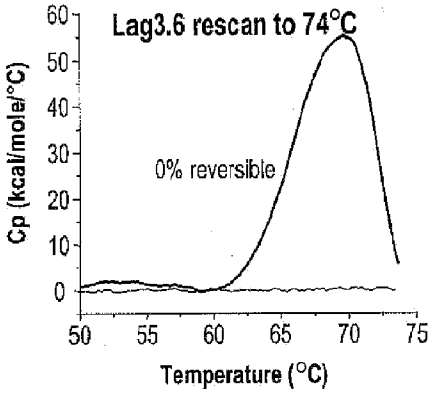


Fig. 6C

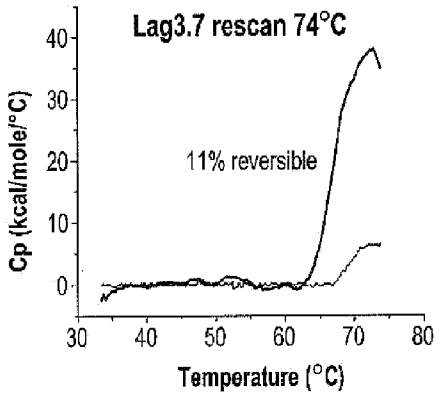

Lag3.7 rescan 74°C
11% reversible
Cp (kcal/mole/°C)
Temperature (°C)
40
30
20
10
0
30
40
50
60
70
80

Fig. 6D

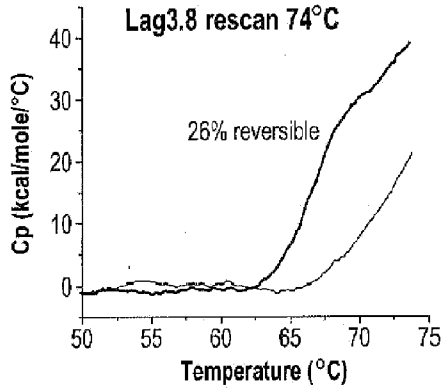

Lag3.8 rescan 74°C
26% reversible
Cp (kcal/mole/°C)
Temperature (°C)
40
30
20
10
0
50
55
60
65
70
75

Fig. 6E

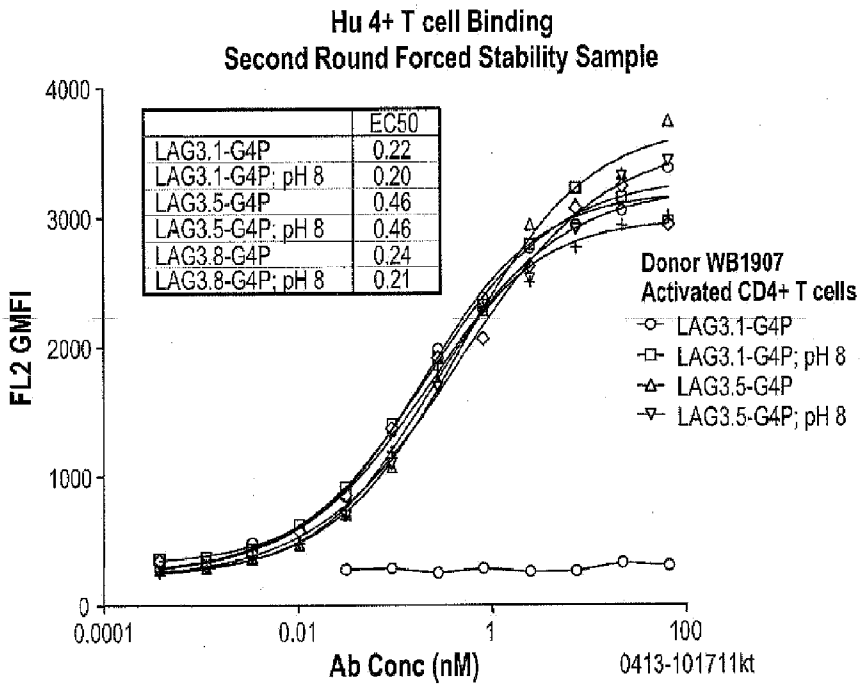

Hu 4+ T cell Binding
Second Round Forced Stability Sample
EC50
LAG3.1-G4P 0.22
LAG3.1-G4P; pH 8 0.20
LAG3.5-G4P 0.46
LAG3.5-G4P; pH 8 0.46
LAG3.8-G4P 0.24
LAG3.8-G4P; pH 8 0.21
Donor WB1907
Activated CD4+ T cells
LAG3.1-G4P
LAG3.1-G4P; pH 8
LAG3.5-G4P
LAG3.5-G4P; pH 8
FL2 GMFI
4000
3000
2000
1000
0
0.0001
0.01
1
100
Ab Conc (nM)
0413-101711kt

Fig. 7

	Mab 5 day incubation	CDR2 peptide alone is shown		CDR2 deamidation
	INHD*GNTNSNPSLK	**INHDGNTNSNPSLK**		
	INHNGNTDSNPSLK		IDHNGNTNSNPSLK	Total
3.1	2.5%	5.1%	1.5%	9%
3.1 pH8	10.0%	15.3%	4.9%	30%

	INHRGSTD?SNPSLK	**INHRGSTNSNPSLK**	ID?HRGSTDSNPSLK	
3.5	2.3%	-	1.5%	4%
3.5 pH8	3.1%	-	1.7%	5%

Fig. 8

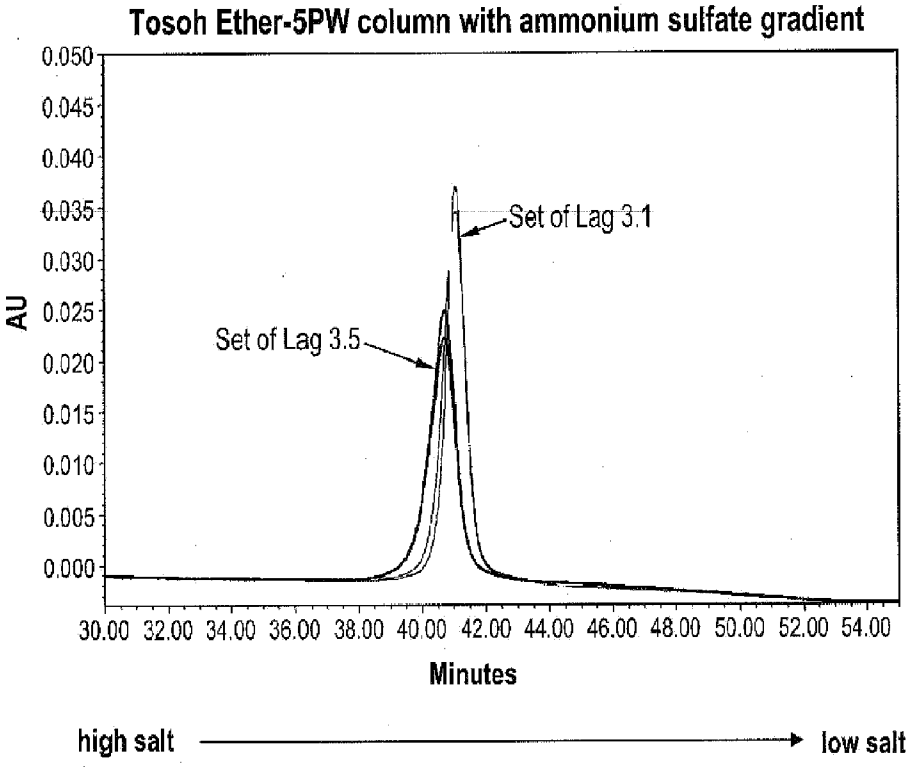

Tosoh Ether-5PW column with ammonium sulfate gradient
AU
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Set of Lag 3.1
Set of Lag 3.5
30.00 32.00 34.00 36.00 38.00 40.00 42.00 44.00 46.00 48.00 50.00 52.00 54.00
Minutes
high salt
low salt

Fig. 9

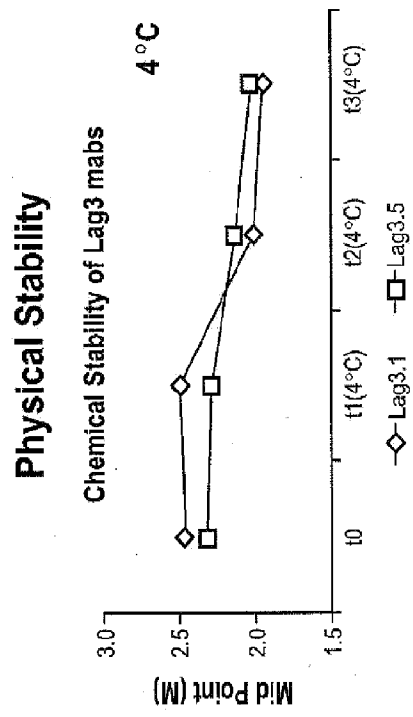


Fig. 10A

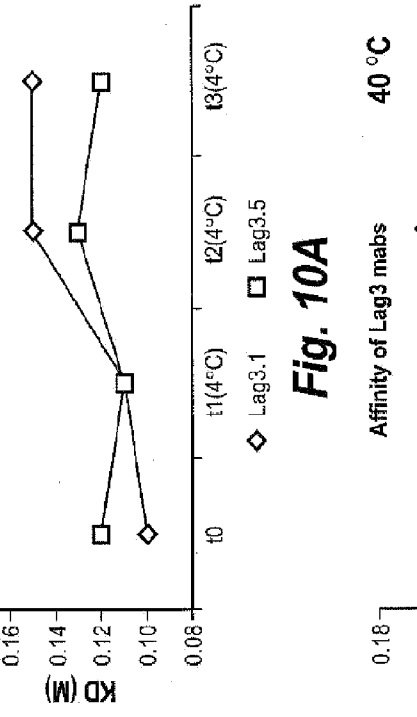


Fig. 10B

Fig. 10C

Fig. 10D

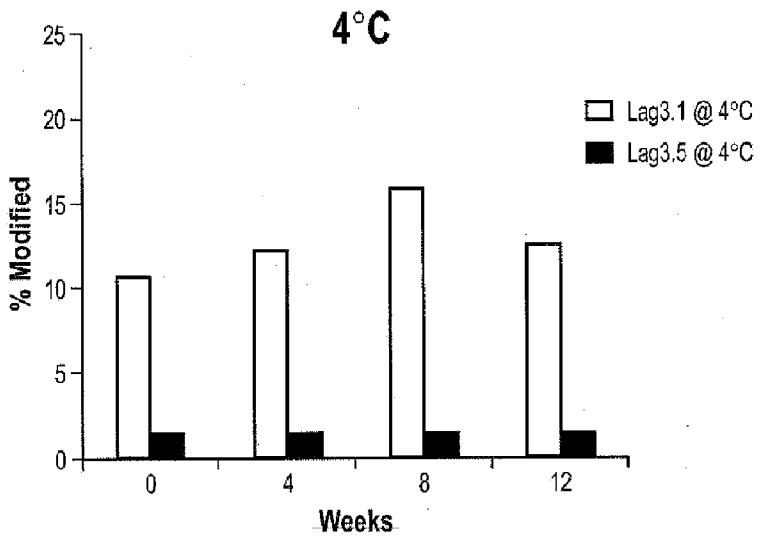


Fig. 11A

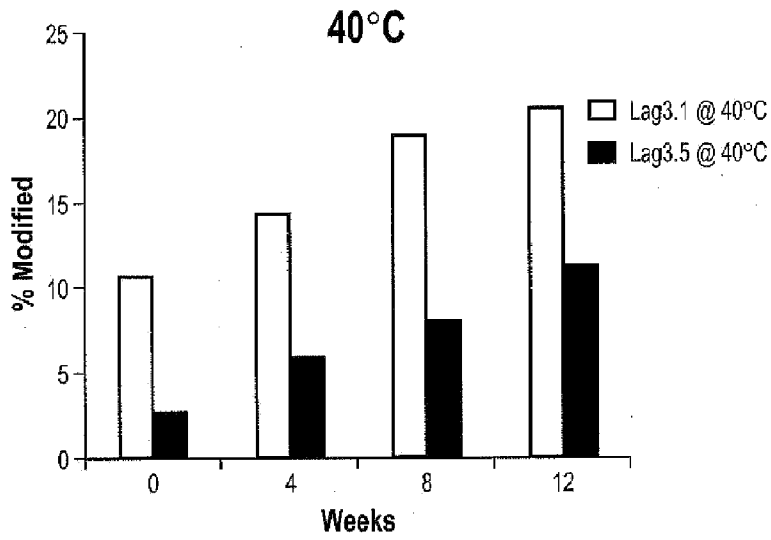


Fig. 11B

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.