

ANTI-KLRG1 ANTIBODIES

TECHNICAL FIELD

[0001] The technical field relates to inhibition of the lymphocyte co-inhibitory
5 receptor Killer Cell Lectin-Like Receptor Subfamily G Member 1 (KLRG1).

BACKGROUND

[0002] Lymphocyte co-inhibitory receptors modulate the action of the adaptive
immune system, for example T cells and NK cells, in response to activating signals such as
antigenic peptides in the context of the major histocompatibility complex (MHC) binding to
10 the T cell receptor (TCR). Co-inhibitory receptors include PD-1, LAG-3, TIM-3, and
CTLA4. The action of co-inhibitory receptors is generally carried out by binding of a ligand
to the extracellular domain of the co-inhibitory receptor followed by recruitment of
intracellular phosphatases by an immunoreceptor tyrosine-based inhibition motif (ITIM)
located in the intracellular domain of the co-inhibitory receptor. The action of co-inhibitory
15 receptors is generally to dampen the immune response of TCR engagement. In recent years it
has been shown that agents that block the activity of co-inhibitory receptors can be used to as
efficacious treatments for cancer and infectious diseases.

[0003] Killer cell lectin-like receptor G1 (KLRG1) is a type II transmembrane protein
acting as co-inhibitory receptor by modulating the activity of T and NK cells. Its extracellular
20 portion contains a C-type lectin domain whose known ligands are cadherins and its
intracellular portion contains an immunoreceptor tyrosine-based inhibitory motif (ITIM)
domain responsible for co-inhibition of T cell receptor (TCR) mediated signaling. KLRG1
ligands can be E-cadherin, N-cadherin, R-cadherin, or a combination thereof.

[0004] The receptor killer cell lectin-like receptor G1 (KLRG1) is expressed on T and NK cells which binds to ligands on epithelial and mesenchymal cells. The ligand for KLRG1 have been described to be E-cadherin, N-cadherin and R-cadherin.

[0005] In humans, KLRG1 expression is confined to cells of the immune systems and specifically to CD8 positive T cells, NK cells and to a lesser extent to CD4 positive T cells. KLRG1 expression has been associated with the late differentiated phenotype. As antigen specific T cells differentiate they acquire increased expression of cytotoxic molecules and therefore have increased cytotoxic potential. The biological function of KLRG1 is to inhibit cytotoxicity and proliferation of these T cells. In cancer and infectious disease, it has been shown beneficial to restore T cell activity.

[0006] In general, a need exists to provide safe and effective therapeutic methods for cancer or infectious diseases. Modulation of the cytotoxic (or CD8+) T and NK cell activation involved in these disorders can be accomplished by manipulation of the KLRG1 pathway.

15 SUMMARY

[0007] The present disclosure provides characterization of novel antibodies, or antigen binding fragments thereof, that bind the extracellular domain (ECD) of KLRG1 and inhibit its interaction with ligands E-cadherin, N-cadherin and R-cadherin. The antibodies here described have been derived by mouse hybridoma technology and can be humanized by grafting their complementary determining regions (CDRs) into a human framework. The disclosure also provides antibodies or binding fragments that modulate (e.g., activate) CD8+ cytotoxic T and NK cells and do so by modulating (e.g., neutralizing) the interaction between KLRG1 and its ligands. The antibodies here described can be used as effective therapeutic agents for treatment of cancer either as monotherapy, or in combination with other

immunotherapy agents (such as anti-PD-1 antibodies, anti-PD-L1 antibodies, or anti-CTLA4 antibodies), or in combination with chemotherapy agents, or cancer vaccines. The antibodies here described can be used as effective treatments for infectious diseases or to enhance the effectiveness of vaccines against infectious diseases.

- 5 [0008] Nonlimiting illustrative embodiments of the antibodies are referred to as ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, or ABC_G1N08. Other embodiments comprise a V_H and/or V_L domain of the Fv fragment of ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, 10 ABC_G1N05, ABC_G1N06, ABC_G1N07, or ABC_G1N08. Further embodiments comprise one or more CDRs of any of these V_H and V_L domains. Other embodiments comprise an H3 fragment of the V_H domain of ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, and ABC_G1N08.
- 15 [0009] The disclosure also provides compositions comprising KLRG1 antibodies, and their use in methods of modulating immune response, including methods of treating humans or animals. In particular embodiments, anti-KLRG1 antibodies are used to treat or prevent cancer by virtue of activating CD8⁺ cytotoxic T and NK cells. Disorders susceptible to treatment with compositions of the invention include but are not limited cancer and infectious 20 diseases.

[0010] Additionally, anti-KLRG1 antibodies may be used diagnostically to detect KLRG1 or its fragments in a biological sample. The amount of KLRG1 detected may be correlated with the expression level of KLRG1, which, in turn, is correlated with the activation status of lymphocytes (e.g., cytotoxic T cells or Natural Killer cells) in the subject.

[0011] The disclosure also provides isolated nucleic acids, which comprise a sequence encoding a V_H or V_L domain from the Fv fragment of ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, and ABC_G1N08. Also provided are isolated
5 nucleic acids, which comprise a sequence encoding one or more CDRs from any of the presently disclosed V_H and V_L domains. The disclosure also provides vectors and host cells comprising such nucleic acids.

[0012] The disclosure further provides a method of producing new V_H and V_L domains and/or functional antibodies comprising all or a portion of such domains derived
10 from the V_H or V_L domains of ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, and ABC_G1N08.

[0013] Additional aspects of the disclosure will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by
15 practicing the invention. The invention is set forth and particularly pointed out in the appended claims, and the present disclosure should not be construed as limiting the scope of the claims in any way. The following detailed description includes exemplary representations of various embodiments of the invention, which are not restrictive of the invention, as claimed. The accompanying figures constitute a part of this specification and, together with
20 the description, serve only to illustrate various embodiments and not limit the invention. Citation of references is not an admission that these references are prior art to the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0014] FIG. 1 shows results of an interferon-gamma (IFN γ) secretion assay demonstrating that anti-KLRG1 antibodies activate CD8⁺ human T cells.

[0015] FIG. 2 shows result of a CD8⁺ T cell proliferation assay demonstrating that anti- KLRG1 antibodies induce proliferation of CD8⁺ T cells.

[0016] FIG. 3 shows relationship between the results of an IFN γ secretion assay in CD8⁺ T cells treated with anti-KLRG1 antibodies and blocking activities (shown in IC₅₀) of
5 the antibodies.

DETAILED DESCRIPTION

Definitions

[0017] The term “antibody,” as used in this disclosure, refers to an immunoglobulin or a fragment or a derivative thereof, and encompasses any polypeptide comprising an
10 antigen-binding site, regardless whether it is produced *in vitro* or *in vivo*. The term includes, but is not limited to, polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, and grafted antibodies. Unless otherwise modified by the term “intact,” as in “intact antibodies,” for the purposes of this disclosure, the term “antibody” also includes antibody fragments such as
15 Fab, F(ab')₂, Fv, scFv, Fd, dAb, and other antibody fragments that retain antigen-binding function, i.e., the ability to bind KLRG1 specifically. Typically, such fragments would comprise an antigen-binding domain.

[0018] The terms “antigen-binding domain,” “antigen-binding fragment,” and “binding fragment” refer to a part of an antibody molecule that comprises amino acids
20 responsible for the specific binding between the antibody and the antigen. In instances where an antigen is large, the antigen-binding domain may only bind to a part of the antigen. A portion of the antigen molecule that is responsible for specific interactions with the antigen-binding domain is referred to as “epitope” or “antigenic determinant.”

[0019] An antigen-binding domain typically comprises an antibody light chain variable region (V_L) and an antibody heavy chain variable region (V_H), however, it does not necessarily have to comprise both. For example, a so-called Fd antibody fragment consists only of a V_H domain, but still retains some antigen-binding function of the intact antibody.

5 [0020] The term “repertoire” refers to a genetically diverse collection of nucleotides derived wholly or partially from sequences that encode expressed immunoglobulins. The sequences are generated by *in vivo* rearrangement of, e.g., V, D, and J segments for H chains and, e.g., V and J segment for L chains. Alternatively, the sequences may be generated from a cell line by *in vitro* stimulation, in response to which the rearrangement occurs. Alternatively,
10 part or all of the sequences may be obtained by combining, e.g., unrearranged V segments with D and J segments, by nucleotide synthesis, randomised mutagenesis, and other methods, e.g., as disclosed in U.S. Pat. No. 5,565,332.

[0021] The terms “specific interaction” and “specific binding” refer to two molecules forming a complex that is relatively stable under physiologic conditions. Specific binding is
15 characterized by a high affinity and a low to moderate capacity as distinguished from nonspecific binding which usually has a low affinity with a moderate to high capacity. Typically, binding is considered specific when the affinity constant K_A is higher than 10^6 M^{-1} or more preferably higher than 10^8 M^{-1} . If necessary, non-specific binding can be reduced without substantially affecting specific binding by varying the binding conditions. The
20 appropriate binding conditions such as concentration of antibodies, ionic strength of the solution, temperature, time allowed for binding, concentration of a blocking agent (e.g., serum albumin, milk casein), etc., may be optimized by a skilled artisan using routine techniques.

[0022] The phrase “substantially as set out” means that the relevant CDR, V_H , or V_L
25 domain of the invention will be either identical to, or have only insubstantial differences in

the specified regions (e.g., a CDR) from the sequence of which is set out. Insubstantial differences include minor amino acid changes, such as substitutions of 1 or 2 out of any 5 amino acids in the sequence of a specified region.

[0023] The term “KLRG1 activity” refers to one or more lymphocyte co-inhibitory activities associated with KLRG1. For example, KLRG1 activity may mean modulation of cytotoxic T and NK cell activation.

[0024] The term “modulate,” and its cognates refer to a reduction or an increase in the activity of KLRG1 associated with activation of T cells and NK cells due to its interaction with an anti-KLRG1 antibody, wherein the reduction or increase is relative to the activity of KLRG1 in the absence of the same antibody. A reduction or an increase in activity is preferably at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more. When KLRG1 activity is reduced, the terms “modulatory” and “modulate” are interchangeable with the terms “inhibitory” and “inhibit.” When KLRG1 activity is increased, the terms “modulatory” and “modulate” are interchangeable with the terms “activating” and “activate.” The activity of KLRG1 can be determined quantitatively using T cell and NK cell activation assays such as those described in Example 6.

[0025] The terms “treatment” and “therapeutic method” refer to both therapeutic treatment and prophylactic/preventative measures. Those in need of treatment may include individuals already having a particular medical disorder as well as those who may ultimately acquire the disorder (i.e., those needing preventative measures).

[0026] The term “effective amount” refers to a dosage or amount that is sufficient to reduce the activity of KLRG1 to result in amelioration of symptoms in a patient or to achieve a desired biological outcome, e.g., reduced activity of KLRG1, modulation of lymphocyte co-inhibition response, increased activation of cytotoxic T and NK cells, or increased release of IFN γ by cytotoxic T cells or NK cells.

[0027] The term “isolated” refers to a molecule that is substantially free of its natural environment. For instance, an isolated protein is substantially free of cellular material or other proteins from the cell or tissue source from which it is derived. The term “isolated” also refers to preparations where the isolated protein is sufficiently pure to be administered as a pharmaceutical composition, or at least 70-80% (w/w) pure, more preferably, at least 80-90% (w/w) pure, even more preferably, 90-95% pure; and, most preferably, at least 95%, 96%, 97%, 98%, 99%, or 100% (w/w) pure.

Anti-KLRG1 Antibodies

[0028] The disclosure provides anti-KLRG1 antibodies that comprise novel antigen-binding fragments.

[0029] In general, antibodies can be made, for example, using traditional hybridoma techniques (Kohler and Milstein (1975) *Nature*, 256: 495-499), recombinant DNA methods (U.S. Pat. No. 4,816,567), or phage display performed with antibody libraries (Clackson et al. (1991) *Nature*, 352: 624-628; Marks et al. (1991) *J. Mol. Biol.*, 222: 581-597). For other antibody production techniques, see also *Antibodies: A Laboratory Manual*, eds. Harlow et al., Cold Spring Harbor Laboratory, 1988. The invention is not limited to any particular source, species of origin, or method of production.

[0030] Intact antibodies, also known as immunoglobulins, are typically tetrameric glycosylated proteins composed of two light (L) chains of approximately 25 kDa each and two heavy (H) chains of approximately 50 kDa each. Two types of light chain, designated as the λ chain and the κ chain, are found in antibodies. Depending on the amino acid sequence of the constant domain of heavy chains, immunoglobulins can be assigned to five major classes: A, D, E, G, and M, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

[0031] The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known in the art. For a review of antibody structure, see Harlow et al., *supra*. Briefly, each light chain is composed of an N-terminal variable domain (V_L) and a constant domain (C_L). Each heavy chain is composed of an N-terminal variable domain (V_H), three or four constant domains (C_H), and a hinge region. The C_H domain most proximal to V_H is designated as C_{H1} . The V_H and V_L domains consist of four regions of relatively conserved sequence called framework regions (FR1, FR2, FR3, and FR4), which form a scaffold for three regions of hypervariable sequence called complementarity determining regions (CDRs). The CDRs contain most of the residues responsible for specific interactions with the antigen. The three CDRs are referred to as CDR1, CDR2, and CDR3. CDR constituents on the heavy chain are referred to as H1, H2, and H3, while CDR constituents on the light chain are referred to as L1, L2, and L3, accordingly. CDR3 and, particularly H3, are the greatest source of molecular diversity within the antigen-binding domain. H3, for example, can be as short as two amino acid residues or greater than 26.

[0032] The Fab fragment (Fragment antigen-binding) consists of the V_H - C_{H1} and V_L - C_L domains covalently linked by a disulfide bond between the constant regions. To overcome the tendency of non-covalently linked V_H and V_L domains in the Fv to dissociate when co-expressed in a host cell, a so-called single chain (sc) Fv fragment (scFv) can be constructed. In a scFv, a flexible and adequately long polypeptide links either the C-terminus of the V_H to the N-terminus of the V_L or the C-terminus of the V_L to the N-terminus of the V_H . Most commonly, a 15-residue (Gly₄Ser)₃ peptide is used as a linker but other linkers are also known in the art.

[0033] Antibody diversity is a result of combinatorial assembly of multiple germline genes encoding variable regions and a variety of somatic events. The somatic events include recombination of variable gene segments with diversity (D) and joining (J) gene segments to

make a complete V_H region and the recombination of variable and joining gene segments to make a complete V_L region. The recombination process itself is imprecise, resulting in the loss or addition of amino acids at the V(D)J junctions. These mechanisms of diversity occur in the developing B cell prior to antigen exposure. After antigenic stimulation, the expressed
5 antibody genes in B cells undergo somatic mutation.

[0034] Based on the estimated number of germline gene segments, the random recombination of these segments, and random V_H - V_L pairing, up to 1.6×10^7 different antibodies could be produced (Fundamental Immunology, 3rd ed., ed. Paul, Raven Press, New York, N.Y., 1993). When other processes which contribute to antibody diversity (such
10 as somatic mutation) are taken into account, it is thought that upwards of 1×10^{10} different antibodies could be potentially generated (Immunoglobulin Genes, 2nd ed., eds. Jonio et al., Academic Press, San Diego, Calif., 1995). Because of the many processes involved in antibody diversity, it is highly unlikely that independently generated antibodies will have identical amino acid sequences in the CDRs.

15 [0035] The disclosure provides novel CDRs derived from human immunoglobulin gene libraries. The structure for carrying a CDR will generally be an antibody heavy or light chain or a portion thereof, in which the CDR is located at a location corresponding to the CDR of naturally occurring V_H and V_L . The structures and locations of immunoglobulin variable domains may be determined, for example, as described in Kabat et al., Sequences of
20 Proteins of Immunological Interest, No. 91-3242, National Institutes of Health Publications, Bethesda, Md., 1991.

[0036] Amino acid sequences of V_H and V_L domains of humanized anti-KLRG1 antibodies are set forth in the Sequence Listing and are enumerated as listed in Tables 1.

Table 1: Amino acid sequences of variable regions for humanized anti-KLRG1 antibodies

SEQ ID NO.	mAb	V _H / V _L	Amino Acid Sequence
SEQ ID NO:1	ABC_H G1N01	V _H	QVILKESGPGLVKPTQTLTLCTFSGFSLTTFG MGIGWIRQPPGKALEWLAHIWWNDDKSYNSA LKSRLTISKDTSKNQVVLMTNMDPVDATY YCARTIYYGNYLTFYAMEHWGQGTTVTVSS
SEQ ID NO:2		V _L	DILMTQSPLSLPVTGPASISCRSSQNIVHSNG NTYLEWYLQKPGQSPRLLIYKVSNRFSGVPDR FSGSGSGTDFTLKISRVEAEDVGVYYCFQGS HPPTFGAGTKLELKRTV
SEQ ID NO:3	ABC_H G1N02	V _H	QVTLKESGPGLVKPTQTLTLCTFSGFSLSTFG MGVGWIRQPPGKALEWLAHIWWDDDKWYEL ALKSRLTISKDSSKNQVVLMTNMDPVDAT YYCARVIYYGSRSAYYSMYWGPGTTVTVSS
SEQ ID NO:4		V _L	DILMTQSPLSLPVTGPASISCKSSQSIVHSNG HTYLEWYLQKPGQSPRLLIYKVSNRFSGVPDR FSGSGSGTDFTLKISRVEAEDVGVYYCFQGS HPVTFGAGTKLELKRTV
SEQ ID NO:5	ABC_H G1N07	V _H	QVQLVESGGGLVKPGGSLRLSCAASGFTFRNY AMSWIRQTPGKGLEWVATISESGNYYNYPDN VKGRLTISRDNKNSLYLQMNLSKAEDTAVY YCVRRDDWEGRAMDYWGQGTTVTVSS
SEQ ID NO:6		V _L	DIQMTQSPSSLSASVGDRVTITCRASRDIGSSL NWYQQKPGGAPKRLIYATSSLDGVPKRFSGS GSGTDFTLTISLQSEDFATYYCLQYASSPWF TGGGTKVEIKRTV

[0037] Particular nonlimiting illustrative embodiments of the antibodies are referred

- 5 to as ABC_HG1N01, ABC_HG1N02, and ABC_HG1N07. Amino acid sequences of CDRs within the V_H and V_L domains of the illustrative embodiments are set forth in the Sequence

Listing and are enumerated as listed in Table 2.

Table 2. CDR amino acid sequences for humanized anti-KLRG1 antibodies

SEQ ID NO.	mAb	CDR	Amino Acid Sequence
SEQ ID NO:7	ABC_HG1 N01	CDR-H1	GFSLTTFGM
SEQ ID NO:8		CDR-H2	WWNDD
SEQ ID NO:9		CDR-H3	TIYYGNYLTFYAMEH
SEQ ID NO:10		CDR-L1	RSSQNIVHSNGNTYLE
SEQ ID NO:11		CDR-L2	KVSNRFS
SEQ ID NO:12		CDR-L3	FQGSHPPT
SEQ ID NO:13	ABC_HG1 N02	CDR-H1	GFSLSTFGM
SEQ ID NO:14		CDR-H2	WWD
SEQ ID NO:15		CDR-H3	VIYYGSRSAYYSMY
SEQ ID NO:16		CDR-L1	KSSQSIVHSNGHTYLE

SEQ ID NO.	mAb	CDR	Amino Acid Sequence
SEQ ID NO:17	ABC_HG1 N07	CDR-L2	KVSNRFS
SEQ ID NO:18		CDR-L3	FQGSHPVPT
SEQ ID NO:19		CDR-H1	GFTFRNY
SEQ ID NO:20		CDR-H2	SESGNY
SEQ ID NO:21		CDR-H3	DDWEGRAMDY
SEQ ID NO:22		CDR-L1	RASRDIGSSLN
SEQ ID NO:23		CDR-L2	ATSSLDS
SEQ ID NO:24		CDR-L3	LQYASSPWT

[0038] Amino acid sequences of V_H and V_L domains of mouse anti-KLRG1

antibodies are set forth in the Sequence Listing and are enumerated as listed in Table 3

Table 3: Variable region amino acid sequences for mouse anti-KLRG1 antibodies

SEQ ID NO.	mAb	V _H / V _L	Amino Acid Sequence
SEQ ID NO:25	ABC_G1 N01	V _H	QVILKESGPGILQPSQTLSTLTCFSFGFSLTTFGMGIGWIR HPSGKALEWLAHIWWNDKSYNSALKSRLTISKDTSKN QVFLRLANVATADTATYYCARTIYYGNYLTFYAMEHW GQGTSVTVSS
SEQ ID NO:26		V _L	DVLLTQTPLSLPVSLGDQASISCRSSQNIVHSNGNTYLE WYLLKPGQSPKLLIFKVSNNRFSGVDPKFSGSGSGTDFTL KIRRVEAEDLGIYYCFQGSHPVPTFGAGTKLELK
SEQ ID NO:27	ABC_G1 N02	V _H	QVTLKESGPGILQPSQTLSTLTCVSGFSLSTFGMGVGVW RQPSGKGLEWLAHIWWDDDKWYELALKSRLTISKDSS KNQVFLKIANVDTADTATYFCARVIYYGSRSAYYSD YWGPSTSVTVSS
SEQ ID NO:28		V _L	DVLMTQTPLSLPVSLGAQASISCKSSQSIVHSNGHTYLE WYLQKPGQSPKILYKVSNNRFSGVDPDRFSGSGSGTDFTL KISRVEAEDLGVIYYCFQGSHPVPTFGAGTKLELK
SEQ ID NO:29	ABC_G1 N03	V _H	QVHLQQSGPELVKPGASVKLSCKASGDTFTTYDITWVK QRPQGGLGWIGWIYPKDGRITQNSEKFKDKATLTVDTSS TTAYMELHSLTSEDSAVYFCARRGQFGPYFDHWGQGS TLTVSS
SEQ ID NO:30		V _L	VIQMTQSSSFLSASLGGRVSITCRASDHIYNWLAWYQQ KPGNAPRLISGATSLETGIPSRFSGGGSGKDYTLTIISLQ TEDIASYYCQYWNTPPTFGGGTKVEIK
SEQ ID NO:31	ABC_G1 N04	V _H	DVQLVESGGGLVQPGGSRKLSAASGFSFSTFGMHWV RQVPEKGLEWVAYISSGSYSIFYADSVKGRFTISRDNPK NTLFLQMTSLRSEDTAIYYCTRTRDSGSSPHYFDYWGQ GTTVTVSS
SEQ ID NO:32		V _L	DIVLTQSPTSLAVSLGQRATISCRASKSVDSYGISFMNW FQKPGQSPKLLIYGASNRGSGVPARFSGSGSGTDFSLN IHPMEEDDTAMYFCQQSKEGPFTFGTGKLELR

SEQ ID NO.	mAb	V _H / V _L	Amino Acid Sequence
SEQ ID NO:33	ABC_G1N05	V _H	EVLLMESGGDLVKPGGSLKLSCAASGFTFSSYDMSWVR QTPDKRLEWVATISSSGRYTFYPDNVKGRFTISRDNK NTLYLQVSNLKSEDTAMYYCSRGTGVTTVVFTDYFDYW GQGTTTLTVSS
SEQ ID NO:34		V _L	DIQMTQSPSSLSASLGERVSLTCRASQDIGNSLNWLQQG PDGTIKRLIYATSSLD SGVPKRFSGSRSGSTYSLTSSLES EDFVAYYCLQYLSSPPTFGGGTKLEIK
SEQ ID NO:35	ABC_G1N06	V _H	QVQLKQSGPGLVQPSQSL SITCTVSGFSLTTHAVHWVR QSPGKGLDWLGVIW SGGNTDYNAAFISRLTISKDNSKS QVFFKMNSLQADDTAIYYCVRLLLPAMDYWGQGTSVT VSS
SEQ ID NO:36		V _L	QIVLTQSPAISASLGERVTMTCTATSSVSSTYLHWYRQ KPGSSPKLWIYSTSTLASGVPVRFGRSGSGTSYSLTSSM EAEDAATYYCHQYRRSPYTFGGGTKLEIK
SEQ ID NO:37	ABC_G1N07	V _H	EVQLVESGGGLVKPGGSLKLSC TASGFTFRNYAMSWV RQTPEKRLEWVATISESGNYNNYPDNVKGRLTISRDNK KNNLYLQMSLLKSEDTAMYYCVRDDWEGRAMDYWG QGTSVTVSS
SEQ ID NO:38		V _L	GIQMTQSPSSLSASLGERVSLTCRASRDIGSSSLNWLQK PDGTIKRLIYATSSLD SGVPKRFSGSRSGTDYSLTSSLES EDFVDYFCLQYASSPWTFGGGTKLEIK
SEQ ID NO:39	ABC_G1N08	V _H	QVQLQQSGAELAKPGASVKLSCKASGYTFTSYFLHWV KQRPQGQLEWIGYMNPSGTYKCNQKFKDKATLTADK SSSTAYMQVSSLTIEDSAVYYCARDRIGYWDFDVWGT GTTVTVSS
SEQ ID NO:40		V _L	DVVMTQSQKFMSTTVGDRVSITCKASQNVGTAVAWY QQKPGQSPKLLIYSASNRYTGVPDRFTGSGSGTDFTLTIT NMQSEDLADYFCQQYSSYLTFGAGTKLIDLK

[0039] Particular nonlimiting illustrative embodiments of the antibodies are referred to as ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05,

ABC_G1N06, ABC_G1N07, and ABC_G1N08. Amino acid sequences of CDRs within the

- 5 V_H and V_L domains of the illustrative embodiments are set forth in the Sequence Listing and are enumerated as listed in Table 4.

Table 4: CDR amino acid sequences for mouse anti-KLRG1 antibodies

SEQ ID NO.	mAb	CDR	Sequence
SEQ ID NO:41	ABC_G1N01	CDR-H1	GFSLTTFGM
SEQ ID NO:42		CDR-H2	WWNDD
SEQ ID NO:43		CDR-H3	TIYYGNYLTFYAMEH
SEQ ID NO:44		CDR-L1	RSSQNIVHSNGNTYLE
SEQ ID NO:45		CDR-L2	KVSNRFS

SEQ ID NO.	mAb	CDR	Sequence
SEQ ID NO:46		CDR-L3	FQGSHPVPT
SEQ ID NO:47		CDR-H1	GFSLSSTFGM
SEQ ID NO:48		CDR-H2	WWDDD
SEQ ID NO:49		CDR-H3	VIYYGSRSAYYSM DY
SEQ ID NO:50		CDR-L1	KSSQSIVHSNGHTYLE
SEQ ID NO:51		CDR-L2	KVSNRFS
SEQ ID NO:52		CDR-L3	FQGSHPVPT
SEQ ID NO:53		CDR-H1	GDTFTTYDIT
SEQ ID NO:54		CDR-H2	YPKDGR
SEQ ID NO:55		CDR-H3	RGQFGPYFDH
SEQ ID NO:56		CDR-L1	RASDHIYNWLA
SEQ ID NO:57		CDR-L2	GATSLET
SEQ ID NO:58		CDR-L3	RGQFGPYFDH
SEQ ID NO:59		CDR-H1	GFSFSTF
SEQ ID NO:60		CDR-H2	SSGSYS
SEQ ID NO:61		CDR-H3	TRTRDSGSSPHYFDY
SEQ ID NO:62		CDR-L1	RASKSVDSYGISFMN
SEQ ID NO:63		CDR-L2	GASNRGS
SEQ ID NO:64		CDR-L3	QQSKEGPFT
SEQ ID NO:65		CDR-H1	GFTFSSY
SEQ ID NO:66		CDR-H2	SSSGRY
SEQ ID NO:67		CDR-H3	TGVTTVVFTDYFDY
SEQ ID NO:68		CDR-L1	SQDIGNS
SEQ ID NO:69		CDR-L2	SSLDS
SEQ ID NO:70		CDR-L3	LQYLSSPPTF
SEQ ID NO:71		CDR-H1	GFSLTTH
SEQ ID NO:72		CDR-H2	WSGGN
SEQ ID NO:73		CDR-H3	LLLPAMDY
SEQ ID NO:74		CDR-L1	TATSSVSSTYLH
SEQ ID NO:75		CDR-L2	STSTLAS
SEQ ID NO:76		CDR-L3	HQYRRSPYT
SEQ ID NO:77		CDR-H1	GFTFRNY
SEQ ID NO:78		CDR-H2	SESGNY
SEQ ID NO:79		CDR-H3	DDWEGRAMDY
SEQ ID NO:80		CDR-L1	RASRDIGSSLN
SEQ ID NO:81		CDR-L2	ATSSLDS
SEQ ID NO:82		CDR-L3	LQYASSPWT
SEQ ID NO:83		CDR-H1	GYTFTSY
SEQ ID NO:84		CDR-H2	NPSSGY
SEQ ID NO:85		CDR-H3	DRIGYWDFDV
SEQ ID NO:86		CDR-L1	KASQNVGTAVA
SEQ ID NO:87		CDR-L2	SASNRYT
SEQ ID NO:88		CDR-L3	QQYSSYLT

[0040] Anti-KLRG1 antibodies may optionally comprise antibody constant regions or parts thereof. For example, a V_L domain may have attached, at its C terminus, antibody light chain constant domains including human C_k or C_λ chains. Similarly, a specific antigen-binding domain based on a V_H domain may have attached all or part of an immunoglobulin heavy chain derived from any antibody isotope, e.g., IgG, IgA, IgE, and IgM and any of the isotope sub-classes, which include but are not limited to, IgG1 and IgG4. In the exemplary embodiments, ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, and ABC_G1N08 antibodies comprise C-terminal fragments of heavy and light chains of human IgG1 λ or IgG1 κ . The DNA and amino acid sequences for the C-terminal fragment of are well known in the art (see, e.g., Kabat et al., Sequences of Proteins of Immunological Interest, No. 91-3242, National Institutes of Health Publications, Bethesda, Md., 1991).

[0041] Certain embodiments comprise a V_H and/or V_L domain of an Fv fragment from ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, and ABC_G1N08. Further embodiments comprise at least one CDR of any of these V_H and V_L domains. Antibodies, comprising at least one of the CDR sequences set out in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, and SEQ ID NO:38 are encompassed within the scope of this invention. An embodiment, for example, comprises an H3 fragment of the V_H domain of antibodies chosen from at least one of ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, and ABC_G1N08.

[0042] In certain embodiments, the V_H and/or V_L domains may be germlined, i.e., the framework regions (FRs) of these domains are mutated using conventional molecular biology techniques to match those produced by the germline cells. In other embodiments, the framework sequences remain diverged from the consensus germline sequences.

5 [0043] In certain embodiments, the antibodies specifically bind an epitope within the ECD of human or mouse KLRG1, with an affinity, as expressed in K_D, of at least about 2 nM, 1nM, 100 pM, 10 pM, or 5 pM. The amino acid sequences of ECDs of human and cynomolgus KLRG1 are set out in SEQ ID NO:89 and SEQ ID NO:90, as listed in Table 6.

[0044] It is contemplated that antibodies of the invention may also bind with other
10 proteins, including, for example, recombinant proteins comprising all or a portion of KLRG1.

[0045] One of ordinary skill in the art will recognize that the antibodies of this invention may be used to detect, measure, and inhibit proteins that differ somewhat from KLRG1. The antibodies are expected to retain the specificity of binding so long as the target protein comprises a sequence which is at least about 60%, 70%, 80%, 90%, 95%, or more
15 identical to any sequence of at least 130, 100, 80, 60, 40, or 20 of contiguous amino acids in the sequence set forth SEQ ID NO:89 or SEQ ID NO:90. The percent identity is determined by standard alignment algorithms such as, for example, Basic Local Alignment Tool (BLAST) described in Altshul et al. (1990) J. Mol. Biol., 215: 403-410, the algorithm of Needleman et al. (1970) J. Mol. Biol., 48: 444-453, or the algorithm of Meyers et al. (1988)
20 Comput. Appl. Biosci., 4: 11-17.

[0046] In addition to the sequence homology analyses, epitope mapping (see, e.g., Epitope Mapping Protocols, ed. Morris, Humana Press, 1996) and secondary and tertiary structure analyses can be carried out to identify specific 3D structures assumed by the disclosed antibodies and their complexes with antigens. Such methods include, but are not
25 limited to, X-ray crystallography (Engstrom (1974) Biochem. Exp. Biol., 11:7-13) and

computer modeling of virtual representations of the presently disclosed antibodies (Fletterick et al. (1986) Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Derivatives

5 [0047] This disclosure also provides a method for obtaining an antibody specific for KLRG1. CDRs in such antibodies are not limited to the specific sequences of V_H and V_L identified in Tables 1 and 3, and may include variants of these sequences that retain the ability to specifically bind KLRG1. Such variants may be derived from the sequences listed in Tables 1 and 3 by a skilled artisan using techniques well known in the art. For example, 10 amino acid substitutions, deletions, or additions, can be made in the FRs and/or in the CDRs. While changes in the FRs are usually designed to improve stability and immunogenicity of the antibody, changes in the CDRs are typically designed to increase affinity of the antibody for its target. Variants of FRs also include naturally occurring immunoglobulin allotypes. Such affinity-increasing changes may be determined empirically by routine techniques that 15 involve altering the CDR and testing the affinity antibody for its target. For example, conservative amino acid substitutions can be made within any one of the disclosed CDRs. Various alterations can be made according to the methods described in Antibody Engineering, 2nd ed., Oxford University Press, ed. Borrebaeck, 1995. These include but are not limited to nucleotide sequences that are altered by the substitution of different codons that 20 encode a functionally equivalent amino acid residue within the sequence, thus producing a “silent” change. For example, the nonpolar amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively 25 charged (acidic) amino acids include aspartic acid and glutamic acid. Substitutes for an amino

acid within the sequence may be selected from other members of the class to which the amino acid belongs (see Table 5). Furthermore, any native residue in the polypeptide may also be substituted with alanine (see, e.g., MacLennan et al. (1998) *Acta Physiol. Scand. Suppl.* 643:55-67; Sasaki et al. (1998) *Adv. Biophys.* 35:1-24).

5 **Table 5: Exemplary conservative substitutions:**

Residue	Conservative substitution
Ala (A)	Ser (S)
Arg (R)	Lys (K)
Asn (N)	Gln (Q); His (H)
Asp (D)	Glu (E)
Cys (C)	Ser (S)
Gln (Q)	Asn (N)
Glu (E)	Asp (D)
Gly (G)	Pro (P)
His (H)	Asn (N), Gln (Q)
Ile (I)	Leu (L), Val (V)
Leu (L)	Ile (I), Val (V)
Lys (K)	Arg (R), Gln (Q)
Met (M)	Leu (L), Ile (I)
Phe (F)	Met (M), Leu (L), Tyr (Y)
Ser (S)	Thr (T); Gly (G)
Thr (T)	Ser (S), Val (V)
Trp (W)	Tyr (Y)
Tyr (Y)	Trp (W), Phe (F)
Val (V)	Ile (I), Leu (L)
Pro (P)	—

[0048] Derivatives and analogs of antibodies of the invention can be produced by various techniques well known in the art, including recombinant and synthetic methods (Maniatis (1990) *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., and Bodansky et al. (1995) *The Practice of Peptide Synthesis*, 2nd ed., Springer Verlag, Berlin, Germany).

10

[0049] In one embodiment, a method for making a V_H domain which is an amino acid sequence variant of a V_H domain of the invention comprises a step of adding, deleting, substituting, or inserting one or more amino acids in the amino acid sequence of the presently

disclosed V_H domain, optionally combining the V_H domain thus provided with one or more V_L domains, and testing the V_H domain or V_H/V_L combination or combinations for a specific binding to KLRG1 and, optionally, testing the ability of such antigen-binding domain to modulate KLRG1 activity. The V_L domain may have an amino acid sequence that is identical
5 or is substantially as set out according to Tables 1 and 3.

[0050] An analogous method can be employed in which one or more sequence variants of a V_L domain disclosed herein are combined with one or more V_H domains.

[0051] A further aspect of the disclosure provides a method of preparing antigen-binding fragment that specifically binds with KLRG1. The method comprises:

- 10 (a) providing a starting repertoire of nucleic acids encoding a V_H domain that either includes a CDR3 to be replaced or lacks a CDR3 encoding region;
- (b) combining the repertoire with a donor nucleic acid encoding an amino acid sequence substantially as set out herein for a V_H CDR3 (i.e., H3) such that the donor nucleic acid is inserted into the CDR3 region in the repertoire, so as to provide a product repertoire of
15 nucleic acids encoding a V_H domain;
- (c) expressing the nucleic acids of the product repertoire;
- (d) selecting a binding fragment specific for KLRG1; and
- (e) recovering the specific binding fragment or nucleic acid encoding it.

[0052] Again, an analogous method may be employed in which a V_L CDR3 (i.e., L3)
20 of the invention is combined with a repertoire of nucleic acids encoding a V_L domain, which either include a CDR3 to be replaced or lack a CDR3 encoding region. The donor nucleic acid may be selected from nucleic acids encoding an amino acid sequence substantially as set out in SEQ ID NOs:7-24 and 41-88.

[0053] A sequence encoding a CDR of the invention (e.g., CDR3) may be introduced
25 into a repertoire of variable domains lacking the respective CDR (e.g., CDR3), using

recombinant DNA technology, for example, using methodology described by Marks et al. (Bio/Technology (1992) 10: 779-783). In particular, consensus primers directed at or adjacent to the 5' end of the variable domain area can be used in conjunction with consensus primers to the third framework region of human V_H genes to provide a repertoire of V_H variable domains lacking a CDR3. The repertoire may be combined with a CDR3 of a particular antibody. Using analogous techniques, the CDR3-derived sequences may be shuffled with repertoires of V_H or V_L domains lacking a CDR3, and the shuffled complete V_H or V_L domains combined with a cognate V_L or V_H domain to make the KLRG1-specific antibodies of the invention. The repertoire may then be displayed in a suitable host system such as the phage display system such as described in WO92/01047 so that suitable antigen-binding fragments can be selected.

[0054] Analogous shuffling or combinatorial techniques are also disclosed by Stemmer (Nature (1994) 370: 389-391), who describes the technique in relation to a β -lactamase gene but observes that the approach may be used for the generation of antibodies.

[0055] In further embodiments, one may generate novel V_H or V_L regions carrying one or more sequences derived from the sequences disclosed herein using random mutagenesis of one or more selected V_H and/or V_L genes. One such technique, error-prone PCR, is described by Gram et al. (Proc. Nat. Acad. Sci. U.S.A. (1992) 89: 3576-3580).

[0056] Another method that may be used is to direct mutagenesis to CDRs of V_H or V_L genes. Such techniques are disclosed by Barbas et al. (Proc. Nat. Acad. Sci. U.S.A. (1994) 91: 3809-3813) and Schier et al. (J. Mol. Biol. (1996) 263: 551-567).

[0057] Similarly, one or more, or all three CDRs may be grafted into a repertoire of V_H or V_L domains, which are then screened for an antigen-binding fragment specific for KLRG1.

[0058] A portion of an immunoglobulin variable domain will comprise at least one of the CDRs substantially as set out herein and, optionally, intervening framework regions from the scFv fragments as set out herein. The portion may include at least about 50% of either or both of FR1 and FR4, the 50% being the C-terminal 50% of FR1 and the N-terminal 50% of FR4. Additional residues at the N-terminal or C-terminal end of the substantial part of the variable domain may be those not normally associated with naturally occurring variable domain regions. For example, construction of antibodies by recombinant DNA techniques may result in the introduction of N- or C-terminal residues encoded by linkers introduced to facilitate cloning or other manipulation steps. Other manipulation steps include the introduction of linkers to join variable domains to further protein sequences including immunoglobulin heavy chain constant regions, other variable domains (for example, in the production of diabodies), or proteinaceous labels as discussed in further detail below.

[0059] Although the embodiments illustrated in the Examples comprise a “matching” pair of V_H and V_L domains, a skilled artisan will recognize that alternative embodiments may comprise antigen-binding fragments containing only a single CDR from either V_L or V_H domain. Either one of the single chain specific binding domains can be used to screen for complementary domains capable of forming a two-domain specific antigen-binding fragment capable of, for example, binding to KLRG1. The screening may be accomplished by phage display screening methods using the so-called hierarchical dual combinatorial approach disclosed in WO92/01047, in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain specific binding domain is selected in accordance with phage display techniques as described.

[0060] Anti-KLRG1 antibodies described herein can be linked to another functional molecule, e.g., another peptide or protein (albumin, another antibody, etc.), toxin,

radioisotope, cytotoxic or cytostatic agents. For example, the antibodies can be linked by chemical cross-linking or by recombinant methods. The antibodies may also be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. No. 4,640,835; 4,496,689;

5 4,301,144; 4,670,417; 4,791,192; or 4,179,337. The antibodies can be chemically modified by covalent conjugation to a polymer, for example, to increase their circulating half-life. Exemplary polymers and methods to attach them are also shown in U.S. Pat. Nos. 4,766,106; 4,179,337; 4,495,285, and 4,609,546.

[0061] The disclosed antibodies may also be altered to have a glycosylation pattern
10 that differs from the native pattern. For example, one or more carbohydrate moieties can be deleted and/or one or more glycosylation sites added to the original antibody. Addition of glycosylation sites to the presently disclosed antibodies may be accomplished by altering the amino acid sequence to contain glycosylation site consensus sequences known in the art. Another means of increasing the number of carbohydrate moieties on the antibodies is by
15 chemical or enzymatic coupling of glycosides to the amino acid residues of the antibody. Such methods are described in WO 87/05330 and in Aplin et al. (1981) CRC Crit. Rev. Biochem., 22: 259-306. Removal of any carbohydrate moieties from the antibodies may be accomplished chemically or enzymatically, for example, as described by Hakimuddin et al. (1987) Arch. Biochem. Biophys., 259: 52; and Edge et al. (1981) Anal. Biochem., 118: 131
20 and by Thotakura et al. (1987) Meth. Enzymol., 138: 350. The antibodies may also be tagged with a detectable, or functional, label. Detectable labels include radiolabels such as ¹³¹I or ⁹⁹Tc, which may also be attached to antibodies using conventional chemistry. Detectable labels also include enzyme labels such as horseradish peroxidase or alkaline phosphatase. Detectable labels further include chemical moieties such as biotin, which may be detected via
25 binding to a specific cognate detectable moiety, e.g., labeled avidin.

[0062] Antibodies, in which CDR sequences differ only insubstantially from those set out in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, or SEQ ID NO:40 are encompassed within the scope of this invention. Typically, an amino acid is substituted by a related amino acid having similar charge, hydrophobic, or stereochemical characteristics. Such substitutions would be within the ordinary skills of an artisan. Unlike in CDRs, more substantial changes can be made in FRs without adversely affecting the binding properties of an antibody. Changes to FRs include, but are not limited to, humanizing a non-human derived or engineering certain framework residues that are important for antigen contact or for stabilizing the binding site, e.g., changing the class or subclass of the constant region, changing specific amino acid residues which might alter the effector function such as Fc receptor binding, e.g., as described in U.S. Pat. Nos. 5,624,821 and 5,648,260 and Lund et al. (1991) J. Immun. 147: 2657-2662 and Morgan et al. (1995) Immunology 86: 319-324, or changing the species from which the constant region is derived.

[0063] One of skill in the art will appreciate that the modifications described above are not all-exhaustive, and that many other modifications would be obvious to a skilled artisan in light of the teachings of the present disclosure.

20 **Nucleic Acids, Cloning and Expression Systems**

[0064] The present disclosure further provides isolated nucleic acids encoding the disclosed antibodies. The nucleic acids may comprise DNA or RNA and may be wholly or partially synthetic or recombinant. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA

molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

[0065] The nucleic acids provided herein comprise a coding sequence for a CDR, a V_H domain, and/or a V_L domain disclosed herein.

5 [0066] The present disclosure also provides constructs in the form of plasmids, vectors, phagemids, transcription or expression cassettes which comprise at least one nucleic acid encoding a CDR, a V_H domain, and/or a V_L domain disclosed here.

[0067] The disclosure further provides a host cell which comprises one or more constructs as above.

10 [0068] Also provided are nucleic acids encoding any CDR (H1, H2, H3, L1, L2, or L3), V_H or V_L domain, as well as methods of making of the encoded products. The method comprises expressing the encoded product from the encoding nucleic acid. Expression may be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression a V_H or V_L domain, or specific binding
15 member may be isolated and/or purified using any suitable technique, then used as appropriate.

[0069] Antigen-binding fragments, V_H and/or V_L domains, and encoding nucleic acid molecules and vectors may be isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially
20 free of nucleic acid or genes of origin other than the sequence encoding a polypeptide with the required function.

[0070] Systems for cloning and expression of a polypeptide in a variety of different host cells are well known in the art. For cells suitable for producing antibodies, see Gene Expression Systems, Academic Press, eds. Fernandez et al., 1999. Briefly, suitable host cells
25 include bacteria, plant cells, mammalian cells, and yeast and baculovirus systems.

Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NS0 mouse myeloma cells, and many others. A common bacterial host is *E. coli*. Any protein expression system compatible with the invention may be used to produce the disclosed antibodies.

- 5 Suitable expression systems include transgenic animals described in Gene Expression Systems, Academic Press, eds. Fernandez et al., 1999.

[0071] Suitable vectors can be chosen or constructed, so that they contain appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors
10 may be plasmids or viral, e.g., phage, or phagemid, as appropriate. For further details see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989. Many known techniques and protocols for manipulation of nucleic acid, for example, in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in
15 detail in Current Protocols in Molecular Biology, 2nd Edition, eds. Ausubel et al., John Wiley & Sons, 1992.

[0072] A further aspect of the disclosure provides a host cell comprising a nucleic acid as disclosed here. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For
20 eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g., vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction of the nucleic acid into the cells may be followed by

causing or allowing expression from the nucleic acid, e.g., by culturing host cells under conditions for expression of the gene.

Methods of Use

[0073] The disclosed anti-KLRG1 antibodies are capable of modulating the KLRG1-associated modulation of the immune responses. In particular embodiments, the activation of cytotoxic T and NK cells is mediated by modulation of KLRG1 signaling. The disclosed antibodies can act as either agonists or antagonists of KLRG1, depending on the method of their use. The antibodies can be used to prevent, diagnose, or treat medical disorders in mammals, especially, in humans. Antibodies of the invention can also be used for isolating KLRG1 or KLRG1-expressing cells. Furthermore, the antibodies can be used to treat a subject at risk of or susceptible to a disorder or having a disorder associated with aberrant KLRG1 expression or function.

[0074] Antibodies of the invention can be used in circumstances where modulation of cytotoxic T and NK cell activation may be desirable, for example, in certain types of cancers and infectious diseases.

[0075] When diminished lymphocyte activation is desirable, the anti-KLRG1 antibodies of the invention may be used as agonists to KLRG1 in order to enhance the KLRG1-associated attenuation of cytotoxic (or CD8+) T and NK cell activation.

[0076] Under certain circumstances, it may be desirable to elicit or enhance a patient's immune response in order to treat cancer or an infectious disease. The disorders being treated or prevented by the disclosed methods include but are not limited to infections with microbes (e.g. bacteria), viruses (e.g., systemic viral infections such as influenza, viral skin diseases such as herpes or shingles), or parasites; and cancer (e.g., melanoma and prostate cancers).

[0077] Cytotoxic T and NK cell activation with anti-KLRG1 antibodies enhances T and NK cell responses. In such cases, antibodies act as antagonists of KLRG1. Thus, in some

embodiments, the antibodies can be used to inhibit or reduce the downregulatory activity associated with KLRG1, i.e., the activity associated with downregulation of cytotoxic T and NK cell activation. As demonstrated in the Examples, a blockade of KLRG1/E-cadherin interaction with antagonizing anti-KLRG1 antibodies leads to enhanced T cell proliferative responses and IFN γ secretion by these cells, consistent with a downregulatory role for the KLRG1 pathway in cytotoxic T and NK cell activation. In various embodiments, the antibodies inhibit binding of E-cadherin to KLRG1 with an IC₅₀ of less than about 50 nM, and more preferably less than about 40, 30, 20, 10, or 5 nM. Inhibition of E-cadherin binding can be measured as described in Example 6 or using techniques known in the art.

10 [0078] The antibodies or antibody compositions of the present invention are administered in therapeutically effective amounts. Generally, a therapeutically effective amount may vary with the subject's age, condition, and sex, as well as the severity of the medical condition of the subject. A therapeutically effective amount of antibody ranges from about 0.001 to about 30 mg/kg body weight, preferably from about 0.01 to about 25 mg/kg body weight, from about 0.1 to about 20 mg/kg body weight, or from about 1 to about 10 mg/kg. The dosage may be adjusted, as necessary, to suit observed effects of the treatment. The appropriate dose is chosen based on clinical indications by a treating physician.

[0079] The antibodies may be given as a bolus dose, to maximize the circulating levels of antibodies for the greatest length of time after the dose. Continuous infusion may also be used after the bolus dose.

[0080] Immune cells (e.g., T cells or NK cells) can also be isolated from a patient and incubated ex vivo with antibodies of the invention. In some embodiments, T cell and NK cell activation can be modulated by removing immune cells from a subject, contacting the immune cells *in vitro* with an anti-KLRG1 antibody of the invention). In such embodiments, the anti-KLRG1 antibody may be used in a multivalent form such that KLRG1 molecules on

the surface of an immune cell become “crosslinked” upon binding to such antibodies. For example, the anti-KLRG1 antibodies can be bound to solid support, such as beads, or crosslinked via a secondary antibody. The immune cells may be then isolated using methods known in the art and reimplanted into the patient.

5 [0081] In another aspect, the antibodies of the invention can be used as a targeting agent for delivery of another therapeutic or a cytotoxic agent (e.g., a toxin) to a cell expressing KLRG1. The method includes administering an anti-KLRG1 antibody coupled to a therapeutic or a cytotoxic agent or under conditions that allow binding of the antibody to KLRG1.

10 [0082] The antibodies of the invention may also be used to detect the presence of KLRG1 in biological samples. The amount of KLRG1 detected may be correlated with the expression level of KLRG1, which, in turn, is correlated with the activation status of immune cells (e.g., activated T cells or NK cells) in the subject.

[0083] Detection methods that employ antibodies are well known in the art and
15 include, for example, ELISA, radioimmunoassay, immunoblot, Western blot, immunofluorescence, immunoprecipitation. The antibodies may be provided in a diagnostic kit that incorporates one or more of these techniques to detect KLRG1. Such a kit may contain other components, packaging, instructions, or other material to aid the detection of the protein.

20 [0084] Where the antibodies are intended for diagnostic purposes, it may be desirable to modify them, for example, with a ligand group (such as biotin) or a detectable marker group (such as a fluorescent group, a radioisotope or an enzyme). If desired, the antibodies of the invention may be labeled using conventional techniques. Suitable detectable labels
25 include, for example, fluorophores, chromophores, radioactive atoms, electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically

detected by their activity. For example, horseradish peroxidase can be detected by its ability to convert tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. For detection, suitable binding partners include, but are not limited to, biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

[0085] Antibodies of the invention can be used in screening methods to identify inhibitors of the KLRG1 pathway effective as therapeutics. In such a screening assay, a first binding mixture is formed by combining KLRG1 and an antibody of the invention; and the amount of binding in the first binding mixture (M0) is measured. A second binding mixture is also formed by combining KLRG1, the antibody, and the compound or agent to be screened, and the amount of binding in the second binding mixture (M1) is measured. A compound to be tested may be another anti-KLRG1 antibody, as illustrated in the Examples. The amounts of binding in the first and second binding mixtures are then compared, for example, by calculating the M1/M0 ratio. The compound or agent is considered to be capable of modulating a KLRG1-associated downregulation of immune responses if a decrease in binding in the second binding mixture as compared to the first binding mixture is observed. The formulation and optimization of binding mixtures is within the level of skill in the art, such binding mixtures may also contain buffers and salts necessary to enhance or to optimize binding, and additional control assays may be included in the screening assay of the invention. Compounds found to reduce the KLRG1-antibody binding by at least about 10% (i.e., $M1/M0 < 0.9$), preferably greater than about 30% may thus be identified and then, if desired, secondarily screened for the capacity to ameliorate a disorder in other assays or animal models as described below. The strength of the binding between KLRG1 and an

antibody can be measured using, for example, an enzyme-linked immunoadsorption assay (ELISA), radio-immunoassay (RIA), surface plasmon resonance-based technology (e.g., Biacore), all of which are techniques well known in the art.

[0086] The compound may then be tested *in vitro* as described in the Examples or in
5 an animal model. Preliminary doses as, for example, determined according to animal tests, and the scaling of dosages for human administration is performed according to art-accepted practices. Toxicity and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The data obtained from the cell culture assays or animal studies can be used in formulating a range of dosage for use in humans.
10 Therapeutically effective dosages achieved in one animal model can be converted for use in another animal, including humans, using conversion factors known in the art (see, e.g., Freireich et al. (1966) Cancer Chemother. Reports, 50(4): 219-244).

Pharmaceutical Compositions and Methods of Administration

[0087] The disclosure provides compositions comprising anti-KLRG1 antibodies.
15 Such compositions may be suitable for pharmaceutical use and administration to patients. The compositions typically comprise one or more antibodies of the present invention and a pharmaceutically acceptable excipient. The phrase “pharmaceutically acceptable excipient” includes any and all solvents, dispersion media, coatings, antibacterial agents and antifungal agents, isotonic agents, and absorption delaying agents, and the like, that are compatible with
20 pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. The compositions may also contain other active compounds providing supplemental, additional, or enhanced therapeutic functions. The pharmaceutical compositions may also be included in a container, pack, or dispenser together with instructions for administration.

[0088] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Methods to accomplish the administration are known to those of ordinary skill in the art. The administration may, for example, be intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous or transdermal. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

[0089] Solutions or suspensions used for intradermal or subcutaneous application typically include one or more of the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol, or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. Such preparations may be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Pharmaceutical compositions suitable for injection include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents,

for example, sugars; polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate, and gelatin.

[0090] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For oral administration, the antibodies can be combined with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches, and the like can contain any of the following ingredients, or compounds of a similar nature; a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0091] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration may be accomplished, for example, through the use of lozenges, nasal sprays, inhalers, or suppositories; For example, in case of antibodies that comprise the Fc portion,

compositions may be capable of transmission across mucous membranes in intestine, mouth, or lungs (e.g., via the FcRn receptor-mediated pathway as described in U.S. Pat. No. 6,030,613). For transdermal administration, the active compounds may be formulated into ointments, salves, gels, or creams as generally known in the art. For administration by

5 inhalation, the antibodies may be delivered in the form of an aerosol spray from pressured container or dispenser, which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[0092] In certain embodiments, the presently disclosed antibodies are prepared with carriers that will protect the compound against rapid elimination from the body, such as a

10 controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. Liposomal suspensions containing the presently disclosed antibodies can also be used as

15 pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[0093] It may be advantageous to formulate oral or parenteral compositions in a dosage unit form for ease of administration and uniformity of dosage. The term “dosage unit form” as used herein refers to physically discrete units suited as unitary dosages for the

20 subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[0094] Toxicity and therapeutic efficacy of the composition of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals,

25 e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the

dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50.

Compositions that exhibit large therapeutic indices are preferred.

[0095] For any composition used in the present invention, the therapeutically
5 effective dose can be estimated initially from cell culture assays. Examples of suitable
bioassays include DNA replication assays, cytokine release assays, transcription-based
assays, KLRG1/cadherin binding assays, immunological assays other assays as, for example,
described in the Examples. The data obtained from the cell culture assays and animal studies
can be used in formulating a range of dosage for use in humans. A dose may be formulated in
10 animal models to achieve a circulating plasma concentration range that includes the IC₅₀
(i.e., the concentration of the antibody which achieves a half-maximal inhibition of
symptoms). Circulating levels in plasma may be measured, for example, by high performance
liquid chromatography. The effects of any particular dosage can be monitored by a suitable
bioassay. The dosage lies preferably within a range of circulating concentrations with little or
15 no toxicity. The dosage may vary depending upon the dosage form employed and the route of
administration utilized.

[0096] The following Examples do not in any way limit the scope of the invention.
One of ordinary skill in the art will recognize the numerous modifications and variations that
may be performed without altering the spirit or scope of the present invention. Such
20 modifications and variations are encompassed within the scope of the invention. The entire
contents of all references, patents, and published patent applications cited throughout this
application are herein incorporated by reference.

EXAMPLES

Example 1: Production of recombinant proteins

- [0097] Recombinant proteins were produced by standard molecular cloning and expression protocols include human KLRG1 ECD (SEQ ID NO:89), cynomolgus KLRG1 ECD (SEQ ID NO:90), and human E-cadherin (SEQ ID NO:91), whose amino acid sequences are shown in Table 6, below. Recombinant proteins were produced as FC fusion or as HIS tagged versions by cloning the respective cDNA into pCDNA4 vector (Invitrogen) and transient transfection in mammalian HEK293. Purification of the expressed proteins took place by chromatography using Protein A affinity resin for the FC fusion versions and Nickel-NTA resin for HIS tagged proteins. All purified proteins were characterized by SDS-PAGE electrophoresis to verify purity and molecular weight.

Table 6: Amino acid sequences of human and cynomolgus KLRG1 ECD and human E-Cadherin

SEQ ID NO.	Protein	Amino Acid Sequence
SEQ ID NO:89	Human KLRG1 ECD	LCQGSNYSTCASCPCPDRWMKYGNHCYYFSVEEKD WNSSLEFCLARDSHLLVITDNQEMSLLQVFLSEAF CWI GLRNNSGWRWEDGSPLNFSRISSNSFVQTCGAINKNGL QASSCEVPLHWVCKKVRL
SEQ ID NO:90	cynomolgus KLRG1 ECD	LCQGSKYSTCASCPCPDHWMKYGNHCYYFSVEKKD WISSLEFCLARDSHLLMITDKQEMSLLQDFLSEAFHWV GLRNNSGWRWEDGSPLNFSRIYSNSLVQTCGAINKNSL QASSCEVSLQWVCKKVSP

SEQ ID NO.	Protein	Amino Acid Sequence
SEQ ID NO:91	Human E-Cadherin	DWVIPPISCPENEGPFPKNLVQIKSNKDKEGKVFYSIT GQGADTPPVGVFIHERETGWLKVTEPLDRERIATYTLFS HAVSSNGNAVEDPMEILITVTDQNDNKPEFTQEVFKGS VMEGALPGTSVMEVTATDADDDVNTYNAAIAYTILSQ DPELPDKNMFTINRNTGVISVTTGLDRESFPTYTLVV QAADLQGEGLSTTATAVITVTDNDNPPIFNPPTYKGG VPENEANVVITTLKVTDADAPNTPAWEAVYTILNDDG GQFVVTNPNVNDGILKTAKGLDFEAKQQYILHVAVT NVVPFEVSLTTSTATVTVDVLDVNEAPIFVPPEKRVEV SEDFGVGQEITSYTAQEPDTFMEQKITYRIWRDTANWL EINPDTGAISTRAELDREDFEHVKNSTYTALIIATDNGS PVATGTGTLILLSDVNDNAPIPEPRTIFFCERNPKPQVI NIIDADLPNTSPFTAELTHGASANWTIQYNDPTQESIIL KPKMALEVGDYKINLKLMDNQNKDQVTTLEVSVDCD EGAAGVCRKAQPVEAGLQIPAILGILGGILALLILLLL LFLRRRAVVKEPLLPEDDTRDNVYYYDEEGGGEEDQ DFDLSQLHRGLDARPEVTRNDVAPTLMSVPRYLPRPA NPDEIGNFIDENLKAADTDPTAPPYDSSLVFDYEGSGSE AASLSSLNSSESDDKDQDYDYLNEWGNRFKKLADMYG GGEDD

- [0098] Stable cell lines were developed to be used as immunization antigens, to test binding of antibodies to full length antigen and as target cells in functional T cell assays. Cell lines developed include CHO expressing full length human KLRG1 and CHO expressing full length cynomolgus KLRG1. Stable cell lines were derived by transfection CHO cells with pCDNA4 plasmid coding protein of interest. After transfection, the cells were exposed to 500ug/ml of G418 to select for stably integrated plasmid. The cell lines were further characterized by FACS for expression and were sorted to select for homogeneous and stable expression.
- 10 [0099] Stable CHO cells double transfected with CD3 agonist and E-cadherin were used in functional assays for demonstrating the effect of blockage of KLRG1 a human T cells. Co-expression of CD3 agonist and E-cadherin was verified by 2 color FACS with appropriate antibodies.

Example 2: Generation of anti-KLRG1 antibodies

[0100] Antibodies ABC_HG1N01, ABC_HG1N02 and ABC_HG1N07 are humanized IgG1 antibodies against the extracellular domain of KLRG1. Mouse monoclonal antibodies (MAB) against human KLRG1 was generated by standard immunizations of female BALB/c mice and SJL mice with human and cynomolgus KLRG1, and subsequent hybridoma screening. Several immunization strategies have been employed to generate a diverse number of antibody hits. Briefly SJL and Balb/c mice were repeatedly immunized with either cDNA, recombinant antigen or CHO cells expressing the antigen of interest. Antigen specific antibody titers were periodically monitored by ELISA and animals were sacrificed when appropriate titers were reached, usually between 1:1000 and 1:10000 dilution factor. Splenocytes from sacrificed mice were fused to mouse myeloma cells to produce hybridoma cells and later cultured and sub-cloned into single cells. Stable clones were scaled up and condition media was harvested and tested for expression of anti-KLRG1 antibodies by ELISA and FACS.

15 **Example 3: Selection of anti-KLRG1 blocking antibodies**

[0101] Hybridoma produced antibodies were screened for binding to human KLRG1 ECD and cynomolgus KRG1 ECD. Antibodies with cross reactivity between both antigens were chosen to move forward to the next stage of screening to determine their ability to neutralize the interaction between KLRG1 and E-cadherin. Antibodies were ranked according to their binding EC50 to human and cyno KLRG1 and further prioritized according to their IC50 in an E-cadherin competition assay. A total of 48 antibodies were selected according to these criteria. Eight antibodies were prioritized for functional characterization in cell-based assay. Table 3 summarizes the variable region amino acid sequences of the 8 mouse antibodies selected and Table 4 shows the CDR regions for the 8 mouse antibodies selected.

25 The antibodies were further ranked according to the presence of sequence liabilities motifs

summarized in Table 7. A deamidation site (NG) was found in CDR-L1 of ABC_G1N01 and ABC_G1N02 that could affect stability manufacturability and activity of drug material. In order to remove this potential liability a series of mutants were constructed and tested. Specifically, the NG motif can be substituted with NA, QG and KG sequences without loss of binding. Mouse antibodies can be humanized by grafting the CDR regions into human framework. Table 1 shows examples of variable region sequences of humanized constructs for ABC_G1N01 (ABC_HG1N01), ABC_G1N02 (ABC_HG1N02) and ABC_G1N07 (ABC_HG1N07). Furthermore conservative mutations can be made to the CDR regions to improve affinity, potency or biophysical characteristics. Table 5 summarizes a list of conservative mutations that can be made to the CDR regions. The monoclonal antibodies were epitope binned according to their ability to compete with each other. It was found that antibodies can be grouped in 3 distinct bins; BIN1 includes: ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05. BIN2 includes: MAB25, MAB031. BIN3 includes: ABC_G1N07, ABC_G1N08. The antibodies were produced as chimeric by cloning the variable mouse regions onto human IgG constant frameworks and tested in functional assays to determine their functional activity on human T cells and described herein.

Table 7: Sequence liability motifs used to screen antibodies for potential manufacturability problems

Liability	Motif	Consequence
Unpaired cysteine	C	Adduct formation, activity loss, scrambling, aggregation, process inconsistency
Deamidation	NG	Activity loss, aggregation, process inconsistency
N-glycosylation	NXS/T, X not P	Impact on PK if heavily sialylated, activity loss
Tyrosine sulphation	Neg-Neg-Y-Neg-Neg	Activity loss, process inconsistency
Hydrolysis Asp-Pro	DP	Fragmentation, stability
Methionine oxidation	M	Activity loss, aggregation
Tryptophan oxidation	W	Photosensitivity, activity loss

Liability	Motif	Consequence
Deamidation	NS, QG, NN	Activity loss, aggregation, process inconsistency
Asparatate isomerisation	DG, DS, DQ, DK	Activity loss, aggregation

Example 4: Characterization of antibody binding to KLRG1 (EC50) and inhibition and KLRG1/E-cadherin interaction by FACS (IC50)

[0102] Binding of anti-KLRG1 monoclonal antibodies to cell expressed human and cynomolgus-KLRG1 was carried out by FACS. Chinese hamster ovary (CHO) cells were stably transfected to express full length human KLRG1 (CHO-human-KLRG1) and cynomolgus KLRG1 (CHO-cynomolgus-KLRG1). EC50 values were determined by incubation of varying concentrations of anti-KLRG1 monoclonal antibodies at concentrations ranging from 1-100nM and measuring fluorescence of the labeled cells using an anti-mouse detection antibody directly conjugated with a fluorescent probe.

[0103] The ability of monoclonal antibodies to inhibit E-cadherin/KLRG1 binding was measured by FACS. Binding of E-cadherin to CHO-hKLRG1 cells was first determined by incubating the KLRG1 expressing cells with varying concentration of HIS tagged recombinant E-cadherin and detected by FACS with anti-HIS detection antibody. EC50 of E-cadherin/KLRG1 interaction was determined by this method to be 1 μ M. IC50 values for each monoclonal antibody was determined by monitoring the loss of E-cadherin binding as a function of varying the concentration of monoclonal antibodies from 0.1 to 100 nM. EC50 and IC50 values are calculated using SigmaPlot software and are summarized in Table 8.

Table 8: Binding EC50 and E-cadherin inhibition IC50 of MABs measured by FACS.

Antibody ID	Binding EC50 (nM)		E-cadherin inhibition IC50 (nM)
	Human KLRG1	Cyno KLRG1	
ABC G1N01	1.21	1.45	12.2
ABC G1N02	1.01	2.02	5.03
ABC G1N03	1.3	1.06	NA
ABC G1N04	29.0	0.47	NA
ABC G1N05	39.8	0.5	23

Antibody ID	Binding EC50 (nM)		E-cadherin inhibition IC50 (nM)
	Human KLRG1	Cyno KLRG1	
ABC_G1N06	1.43	1.5	41
MAB031	5.1	5.93	39
ABC_G1N07	0.94	0.98	9.6
ABC_G1N08	4.8	1.3	10

Example 5: Measurement of binding kinetics

[0104] Binding kinetics of humanized antibodies for human and cynomolgus KLRG1 were determined by OCTET® measurement. OCTET® Systems use Bio-Layer Interferometry (BLI) technology to monitor the binding of proteins and other biomolecules to their partners directly in real time, providing analysis of kinetic binding constants. *See* <https://www.fortebio.com/bli-technology.html>, the contents of which, including all links and sublinks associated therewith, is incorporated by reference herein in its entirety. The experimental set-up consists of immobilizing biotinylated recombinant antigen (Human-KLRG-ECD or cynomolgus-KLRG1-ECD) on streptavidin OCTET® sensor to produce antigen loaded sensors. The loaded sensors are the exposed to varying concentrations of each humanized antibody from 100 to 0.1 nM in the OCTET® instruments and data collected for 600 seconds to measure association kinetic (K_{on}) of the antibody/antigen complex. In a following step the sensors are exposed to a solution of 1 X phosphate buffered saline (PBS) buffer devoid of antibody for 600 seconds to observe dissociation kinetics (K_{off}). The resulting data is then fitted to 1:1 binding kinetics model using ForteBio® analysis software to calculate the K_D . Kinetic binding parameters derived by this method are summarized in Table 9 for the three humanized antibodies.

20 **Table 9: Binding affinity of selected MABs measured by OCTET®:**

Antigen	Antibody	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
Human KLRG1	ABC_G1N01	2.29E+06	2.87E-04	1.25E-10
	ABC_G1N02	2.80E+06	6.81E-04	2.43E-10

Antigen	Antibody	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
	ABC_G1 N07	1.97E+06	<1.0E-05	<5.1E-12
Cyno KLRG1	ABC_G1 N01	6.64E+05	4.10E-05	6.17E-11
	ABC_G1 N02	6.30E+05	4.07E-05	6.47E-11
	ABC_G1 N07	8.80E+05	1.25E-03	1.42E-09

Example 6: Characterization of functional activity for KLRG1 blocking antibodies

[0105] Blocking KLRG1 interaction with its ligands has an activating effect on human CD8⁺ T cell by measuring the effect of antibody mediated blockade on KLRG1 signaling on production of IFN γ and on the proliferation of T cells (Proliferation index). To demonstrate the effect of KLRG1 blockade on cells of the immune system, CD8⁺ T cells were isolated from healthy donors and tested in co-culture with a CHO cell line co-expressing a CD3 agonist and a E-cadherin (eAPC). The assay works by providing T cells with 2 competing signals where CD3 stimulation is counteracted by the inhibitory effect of E-cadherin. When KLRG1 signaling is blocked by anti-KLRG1 antibodies, the inhibitory signal is disrupted, and T cells are activated according to their interaction with the CD3 agonist expressed on the cell surface. IFN γ secretion is measured by ELISA and results summarized in Table 10 and FIG 1.

Table 10: Restoration of IFN γ Release by KLRG1 blocking antibodies in Human CD8⁺ T cells

	IFN γ released (pg/ml)	
	Sample 1	Sample 2
CD8 positive control	1624.09	1401.42
Isotype	390.02	355.94
ABC_G1N07	1579.71	1389.6
ABC_G1N02	1440.87	1290.52
ABC_G1N01	1185.14	1217.73
ABC_G1N06	1023.15	1142.09
ABC_G1N05	1012.46	1083.04
ABC_G1N08	1013.8	1063.23

	IFN γ released (pg/ml)	
	Sample 1	Sample 2
CD8 positive control	1624.09	1401.42
ABC_G1N04	987.48	1002.46
ABC_G1N03	934.34	868.9

[0106] This data demonstrate that E-cadherin has an inhibiting effect on human T cells which can be reversed by blockade of KLRG1 signaling with blocking antibodies.

[0107] The data presented in Table 11 and FIG 2 demonstrates that blocking of KLRG1/E-cadherin interaction results in proliferation of human CD8⁺ T cells by measuring the proliferation index in response to eAPC. In this assay eAPC were produced by stably transducing CHO cells with h-Ecadherin and an CD3 agonist agent. The eAPC thus produced were then co-incubated for 3 days with freshly isolated CD8⁺ T cells from a healthy volunteer in the presence of 10 micro-gram/ml of test antibodies or isotype control. The positive control sample was prepared by incubating CD8⁺ T cells with CHO cells stably expressing anti-CD3 but lacking expression of inhibitory ligand E-cadherin, thus allowing un-inhibited stimulation of CD8⁺ T cell by anti-CD3 expressed on CHO cells. The results show that CD8⁺ T cells proliferate in response to CD3 stimulation as expected and that the inhibitory ligand E-cadherin co-expression on eAPC inhibits proliferation of CD8⁺ T cells. Furthermore, CD8⁺ T cell proliferation can be restored by blockade of KLRG1 by neutralizing antibodies.

TABLE 11: Effect of KLRG1 blockage on CD8⁺ T cell proliferation

	Division Index	
Positive Control	0.214	0.229
Isotype	0.139	0.121
ABC_G1N07	0.252	0.235
ABC_G1N02	0.25	0.236

[0108] The data presented in Table 12 and FIG 3 shows the correlation between T cell activity in the IFN γ secretion assay and blocking activity of anti-KLRG1 neutralizer

antibodies. IC50 values for each antibody were derived from E-cadherin binding inhibition studies and plotted against IFN γ production levels measured in CD8 $^{+}$ T cells IFN γ release assays. The correlation shows that antibodies with lower IC50 values (e.g. more potent blockers of KLRG1/E-cadherin binding) result in higher levels of IFN γ release by CD8 $^{+}$ T cells. This data demonstrates that antibody blockade of KLRG1 results in restoration of T cell activity in an E-cadherin dependent manner.

Table 12: Correlation between E-cadherin IC50 competition values and IFN γ Secretion

mAb	IFNγ (pg/ml)	IC50 (nM)
ABC G1N07	1512.76	4.21
ABC G1N02	1484.66	9.6
ABC G1N01	1365.7	5
MAB034	1201.44	11.2
MAB024	1129.8	15.4
ABC G1N05	1082.62	12.3
ABC G1N08	1047.75	23
MAB036	1038.52	10
MAB031	975.34	18
Isotype	799.27	39

CLAIMS

What is claimed is:

1. An antibody, or antigen binding fragment thereof, comprising an amino acid sequence as set out in SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:21, SEQ ID NO:43, SEQ ID NO:49,
5 SEQ ID NO:55, SEQ ID NO:61, SEQ ID NO:67, SEQ ID NO:73, SEQ ID NO:79, or SEQ ID NO:85.
2. The antibody of claim 1, wherein said antibody specifically binds to the extracellular domain of human Killer Cell Lectin-Like Receptor Subfamily G Member 1 (KLRG1) and the extracellular domain cynomolgus monkey KLRG1.
- 10 3. The antibody of claim 1, comprising an amino acid sequence substantially as set out in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, or SEQ ID NO:40.
- 15 4. The antibody of claim 1, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, and SEQ
20 ID NO:40.
5. The antibody of claim 1, wherein the antibody specifically binds to an amino acid sequence that is at least 95% identical to any sequence of at least 100 of contiguous amino acids of at least one sequence selected from group consisting of SEQ ID NO:89 and SEQ ID NO:90.

6. The antibody of claim 5, wherein the antibody specifically binds to the extracellular domain of KLRG1 with an affinity, as expressed in K_D , of at least about 2 nM, about 1 nM, about 100 pM, about 10 pM, or about 5 pM.
7. The antibody of claim 5, where the antibody inhibits the binding of E-cadherin to
5 KLRG1 with an IC₅₀ of less than about 50 nM, 40 nM, 30 nM, 20 nM, or 10 nM.
8. The antibody of claim 1, wherein the antibody is humanized.
9. The antibody of claim 1, wherein the antibody is IgG₁ or IgG₄.
10. The antibody of claim 9, wherein the antibody is IgG_{1λ} or IgG_{1κ}.
11. The antibody of claim 1, wherein the antibody is ABC_HG1N01, ABC_HG1N02,
10 ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, or ABC_G1N08.
12. A pharmaceutical composition comprising the antibody of claim 1 and a pharmaceutically acceptable carrier.
13. A method of treatment comprising administering an effective dose of the
15 pharmaceutical composition of claim 12.
14. The method of claim 13, wherein the pharmaceutical composition is administered to a subject in need for treatment or prevention of cancer or an infectious disease.
15. The method of claim 13, wherein the subject is a human.
16. An antibody comprising human framework regions, wherein said antibody
20 specifically binds KLRG1, and wherein the antibody is capable of blocking binding between human or cynomolgus monkey KLRG1 and E-cadherin.
17. The antibody of claim 16, wherein said antibody comprises a CDR derived from ABC_HG1N01, ABC_HG1N02, ABC_HG1N07, ABC_G1N01, ABC_G1N02, ABC_G1N03, ABC_G1N04, ABC_G1N05, ABC_G1N06, ABC_G1N07, or ABC_G1N08.
- 25 18. An isolated nucleic acid encoding the antibody of claim 1.

19. An expression vector comprising the nucleic acid of claim 18.
20. A host cell comprising the vector of claim 19.
21. The host cell of claim 20, wherein the host cell is chosen from: an *E. Coli* bacterium, a Chinese hamster ovary cell, a HeLa cell, and a NS0 cell.
- 5 22. The nucleic acid of claim 18, wherein the nucleic acid encodes the amino acid sequence set out in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, or
10 SEQ ID NO:40.
23. A method of making an antibody, or fragment thereof, that specifically binds with the extracellular domain of human and cynomolgus monkey KLRG1, the method comprises:
- (a) providing a starting repertoire of nucleic acids encoding a variable domain that either includes a CDR3 to be replaced or lacks a CDR3 encoding region;
- 15 (b) combining the repertoire with a donor nucleic acid encoding an amino acid sequence substantially as set out in SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:21, SEQ ID NO:43, SEQ ID NO:49, SEQ ID NO:55, SEQ ID NO:61, SEQ ID NO:67, SEQ ID NO:73, SEQ ID NO:79, or SEQ ID NO:85, such that the donor nucleic acid is inserted into the CDR3 region in the repertoire, so as to provide a product repertoire
20 of nucleic acids encoding a variable domain;
- (c) expressing the nucleic acids of the product repertoire;
- (d) selecting nucleic acid from step (c) encoding a variable domain, or fragment thereof, which specifically binds said extracellular domain of human and cynomolgus monkey KLRG1; and

(e) recovering the variable domain, or fragment thereof, or nucleic acid encoding said variable domain or fragment thereof of (d).

24. An antibody produced by the method of claim 23.
25. A method of modulating CD8⁺ T and NK cell activation comprising contacting a
5 lymphocyte with an anti-KLRG1 antibody, or fragment thereof.
26. The method of claim 25, wherein the lymphocyte is a T cell or a NK cell.
27. The method of claim 25, wherein the antibody is as in claim 1.
28. The method of claim 25, wherein the antibody is as in claim 24.
29. The method of claim 25, wherein the anti-KLRG1 antibody modulates CD8⁺ T and
10 NK activation and inhibits the binding of E-cadherin to human and cynomolgus monkey
KLGR1.
30. An antibody, or antigen binding fragment thereof, which cross-competes with the
antibody of claim 1.
31. A composition comprising the antibody of claim 1.