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(54) Title: ANTI-VISTA ANTIBODIES AND FRAGMENTS

(57) Abrégé/Abstract:

The present invention relates to novel antibodies and fragments that bind to a V- domain Ig Suppressor of T cell Activation (VISTA), and methods of detecting VISTA.

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(54) Title: ANTI-VISTA ANTIBODIES AND FRAGMENTS

(57) Abstract: The present invention relates to novel antibodies and fragments that bind to a V- domain Ig Suppressor of T cell Activation (VISTA), and methods of detecting VISTA.

ANTI-VISTA ANTIBODIES AND FRAGMENTS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/184,108, filed on June 24, 2015, and U.S. Provisional Application No. 62/187,659, filed on July 1, 2015.

[0002] This application includes the Sequence Listing contained in the following ASCII text file being submitted concurrently herewith:

a) File name: 01481142017SEQUENCELISTING.txt; created June 22, 2016, 87 KB in size.

BACKGROUND OF THE INVENTION

[0003] The expression of negative immune checkpoint regulators by cancer cells or immune cells in the tumor microenvironment can suppress the host's immune response against the tumor. To effectively combat the cancer, it is desirable to block tumor-mediated suppression of the host immune response. Accordingly, there is a need for new and effective therapeutic agents that inhibit negative immune checkpoint regulators in the tumor microenvironment that suppress anti-tumor immune responses.

SUMMARY OF THE INVENTION

[0004] The present invention provides, in one embodiment, an isolated antibody (*e.g.*, a chimeric antibody), or antigen-binding fragment thereof, comprising an antigen-binding region that binds to a mammalian V-domain Ig Suppressor of T cell Activation (VISTA) protein. The antibody comprises an antibody VH domain comprising a VH CDR1 having the amino acid sequence of SEQ ID NO:31, a VH CDR2 having the amino acid sequence of SEQ ID NO:32 and a VH CDR3 having the amino acid sequence of SEQ ID NO:33. The antibody further comprises an antibody VL domain comprising a VL CDR1 having the amino acid sequence of SEQ ID NO:34, a VL CDR2 having the amino acid sequence of SEQ ID NO:35

and a VL CDR3 having the amino acid sequence of SEQ ID NO:36. In addition, the antibody comprises a non-human antibody heavy chain constant region and a non-human antibody light chain constant region.

[0005] In one embodiment, the antibody VH domain comprises SEQ ID NO:64. In another embodiment, the antibody VL domain comprises SEQ ID NO:45.

[0006] In some embodiments, the antibody comprises a non-human antibody heavy chain constant region that is a murine antibody heavy chain constant region. In a particular embodiment, the murine antibody heavy chain constant region is a murine IgG1 heavy chain constant region. In a specific embodiment, the murine IgG1 heavy chain constant region comprises the heavy chain constant region in SEQ ID NO:76. In another embodiment, the murine antibody heavy chain constant region is a murine IgG2a heavy chain constant region.

[0007] In other embodiments, the antibody comprises a non-human antibody light chain constant region that is a murine antibody light chain constant region. In a particular embodiment, the murine antibody light chain constant region is a murine IgG1 light chain constant region. In a specific embodiment, the murine IgG1 light chain constant region comprises the light chain constant region in SEQ ID NO:77. In another embodiment, the murine antibody light chain constant region is a murine IgG2a light chain constant region.

[0008] In a certain embodiment, the antibody or antigen-binding fragment binds to a human VISTA protein. In a particular embodiment the antibody or antigen-binding fragment binds to an epitope that is present in a human VISTA protein having the amino acid sequence shown below:

MGVPTALEAGSWRWGSLLFALFLAASLGPVAAFKVATPYSLYVCPEGQNVTLTCRL
LGPVDKGHDVTFYKTWYRSSRGEVQTCSERRPIRNLTFQDLHLHHGGHQAANTSHD
LAQRHGLESASDHHGNFSITMRNLTLLDSGLYCCLVVEIRHHHSEHRVHGAMELQV
QTGKDAPSNCVVYPSSSQESENITAAALATGACIVGILCLPLILLLVYKQRQAASNRR
AQELVRMDSNIQGIENPGFEASPPAQGIPEAKVRHPLSYVAQRQPSESGRHLLSEPSTP
LSPPGPGDVF (SEQ ID NO:46).

[0009] In one embodiment, the antibody or antigen-binding fragment is a whole antibody.

[0010] In another embodiment, the invention provides an isolated antibody, or antigen-binding fragment thereof, comprising an antibody heavy chain comprising SEQ ID NO:77 and an antibody light chain comprising SEQ ID NO:68.

[0011] In yet another embodiment, the invention provides a composition comprising an antibody or antigen-binding fragment of the invention.

[0012] In an additional embodiment, the invention provides a method for detecting a mammalian VISTA protein in a sample. The method comprises contacting the sample with an antibody or antigen-binding fragment of the invention under conditions in which the antibody or antigen-binding fragment binds to VISTA protein in the sample, and detecting the antibody or antigen-binding fragment that is bound to VISTA protein in the sample.

[0013] In one embodiment, the sample comprises cells. The cells can include immune cells (*e.g.*, myeloid cells), stromal cells (*e.g.*, fibroblasts, endothelial cells), and/or cancer (*e.g.*, malignant) cells, such as lung cancer cells, prostate cancer cells, acute myeloid leukemia (AML) cells, melanoma cells, ovarian cancer cells or colon cancer cells, or any combination thereof, among others. In a particular embodiment, the sample comprises a tissue or biological fluid.

[0014] In one embodiment, the antibody or antigen-binding fragment employed in the method comprises a detectable label.

[0015] In a certain embodiment, the method includes an immunohistochemical (IHC) staining assay. In another embodiment, the method includes a flow cytometry assay.

[0016] The present invention provides improved reagents and methods for detecting VISTA protein(s) in biological materials. Such reagents and methods typically have enhanced sensitivity and/or specificity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figures 1A-1C: Graphs showing VISTA expression on TF1 AML Cells Expression of VISTA protein by flow cytometry is shown in the TF-1 AML cell line.

[0018] Figures 2A-2E: Graphs showing staining and gating strategies for identification of Human Myeloid and Lymphoid Subsets.

[0019] Figures 3A-3G: Graphs showing expression of VISTA on Human Myeloid and Lymphoid Subsets from one healthy normal donor.

[0020] Figure 4: Graph showing expression of VISTA on Human Myeloid and Lymphoid Subsets across multiple healthy normal donors.

[0021] Figures 5A-5B: Graph showing staining and gating strategies for identification of expression of VISTA on Human Monocytes and Macrophages.

[0022] Figures 6A-6C: Graphs showing expression of VISTA on Human Monocytes and Macrophages.

[0023] Figures 7A-7E: Graphs showing staining and gating strategies for identification of expression of VISTA on Human T and NK Cell Subsets.

[0024] Figures 8A-8G: Graphs showing expression of VISTA on Human T and NKCell Subsets from one healthy normal donor.

[0025] Figure 9: Graph showing expression of VISTA on Human T and NK Cell Subsets across multiple healthy normal donors.

[0026] Figures 10A-10D: Graphs showing staining and gating strategies for identification of expression of VISTA on Human Dendritic Cell subsets.

[0027] Figures 11A-11C: Graphs showing expression of VISTA on Human Dendritic Cell subsets and basophils from one healthy normal donor.

[0028] Figure 12: Graph showing expression of VISTA on Human Dendritic Cell Subsets and basophils across multiple healthy normal donors.

[0029] Figures 13A-13D: Analysis of VISTA expression on healthy human peripheral blood cells. Profile of VISTA expression on healthy human peripheral blood cells using multicolor flow cytometry analysis: Whole blood samples from 2 different individuals were analyzed for VISTA expression on (Figure 13A) monocytes $SSC^{lo,}CD11b^{hi}CD14^{hi}CD16^{-ve}CD33^{+ve}HLA\text{-}DR^{+ve}CD19^{-ve}$) (Figure 13B) neutrophils ($SSC^{hi}CD177^{+}CD11b^{hi}CD14^{lo}CD16^{+ve}CD33^{+ve}HLA\text{-}DR^{-ve}CD19^{-ve}$). Peripheral blood mononuclear cells were isolated using Ficoll gradient for analysis of (Figure 13C) CD4+ T cells ($CD3^{+ve}CD4^{+ve}$), and (Figure 13D) CD8+ T cells ($CD3^{+ve}CD8^{+ve}$).

[0030] Figures 14A-14C: Analysis of VISTA expression on peripheral blood cells from a lung cancer patient and a healthy control donor. Profile of VISTA expression on lung cancer patient peripheral blood cells using multicolor flow cytometry analysis: Representative FACS plot (Figure 14A) from one individual is shown. Peripheral blood mononuclear cells were isolated by Ficoll and analyzed for VISTA expression on (Figure 14B) monocytes (CD14+ CD11b+ CD33+ HLADR+ CD15-) and (Figure 14C) myeloid derived suppressor cells (CD14- CD11b+ CD33-HLADR-CD15+ CD16+).

[0031] Figures 15A-15C: Profile of VISTA expression in peripheral blood cells from a patient with colon cancer, using multicolor flow cytometry analysis: Representative FACS plot (Figure 15A) from one individual is shown. Peripheral blood mononuclear cells were isolated by Ficoll and analyzed for VISTA expression on (Figure 15B) monocytes (CD14+ CD11b+ CD33+ HLADR+ CD15-) and (Figure 15C) myeloid derived suppressor cells (CD14- CD11b+ CD33-HLADR-CD15+ CD16+).

[0032] Figures 16A-16D: Profile of VISTA expression on Cynomolgus monkey peripheral blood cells using multicolor flow cytometry analysis: Whole blood from 4 different monkeys was analyzed for VISTA expression on (Figure 16A) monocytes ($SSC^{lo}CD11b^{hi}CD14^{hi}HLA\text{-}DR^{hi}CD16^{-ve}CD19^{-ve}$ and (Figure 16B) neutrophils $CD11b^{hi}CD14^{lo}HLA\text{-}DR^{-ve}CD16^{-ve}CD19^{-ve}$. Peripheral blood mononuclear cells from three monkeys were isolated using Ficoll gradient for analysis of (Figure 16C) CD4+ T cells ($TCR\alpha/\beta^{+ve}CD4^{+ve}$) and (Figure 16D) CD8+ T cells ($TCR\alpha/\beta^{+ve}CD8^{+ve}$) .

[0033] Figure 17: Graph showing absolute expression values of VISTA RNA in Heme cell lines.

[0034] Figure 18: Mouse A20 cells were stably transfected with either GFP or human VISTA. They were incubated with ova peptide and with DO11.10 T cells. CD25 expression by the T cells was measured 24 hours after incubation began. The A20-huVISTA cells suppress CD25 expression by the T cells, but this readout is significantly restored by incubation with VSTB95.

[0035] Figures 19A-19F: Graphs showing Human VISTA ELISA results.

[0036] Figures 20A-20F: Human VISTA FACS results, showing anti-VISTA antibodies binding to cells expressing human VISTA.

[0037] Figures 21A-21D: Dilution study of 6 anti-VISTA antibody candidates in the mixed lymphocyte reaction from 30μg/ml to 0.0μg/ml.

[0038] Figures 22A-22B: Dilution studies of 6 anti-VISTA antibody candidates in the SEB assay (individual CPM counts and IFN-g concentrations) from 30μg/ml to 0.0μg/ml.

[0039] Figure 23: Sensorgram plot using anti-VISTA antibody VSTB85 coated on a Proteon SPR chip and VISTA protein with the indicated competitors run over the chip (competitors listed in Table 16).

[0040] Figure 24: Experimental design for MB49 murine bladder tumor model

[0041] Figures 25A-25B: MB49 tumor growth in female C57Bl/6 mice. Graphs illustrate tumor growth in individual mice treated with anti-mouse VISTA antibody (Figure 25B) or control IgG (Figure 25A).

[0042] Figure 26: Amino acid sequence of human VISTA (SEQ ID NO:46).

[0043] Figure 27: Multiple sequence alignment of VISTA orthologues

[0044] Figure 28: Regions of human VISTA bound by VSTB50 and VSTB60 antibodies (top) or VSTB95 and VSTB112 antibodies (bottom), as determined by HDX

[0045] Figure 29: VISTA Epitope bound by VSTB112. (Top) VISTA is shown in cartoon with strands labeled. Residues having at least one atom within 5 Å of VSTB112 in the complex are colored blue. Blue and orange spheres highlight a chain break, and the cyan and green spheres mark the N- and C-termini of the VISTA structure, respectively. (Bottom) Sequence of VISTA construct used in structure determination. Circles below the sequence are used to indicate residues which make only main chain contacts with VSTB112, triangles indicate a side chain contact, and squares indicate the side chain contact results in either a hydrogen bond or salt bridge interaction as calculated by PISA. Shapes are colored to indicate the CDR having the greatest number of atoms contacted by the given residue with CDR colors defined in Figure 59. Secondary structural elements are as defined in the program MOE with yellow arrows representing β-strands and red rectangles indicating α-helices.

[0046] Figure 30: VSTB112 Paratope. (Top) VISTA antigen is shown in illustration and VSTB112 within 5 angstrom (Å) of VISTA is shown in surface with colors used to designate CDR identity as specified in the sequence below. Contacting framework residues adjacent to a CDR are colored similarly to the corresponding CDR (Bottom) Sequence of VSTB112 Fv region. Colored backgrounds specify CDRs according to Kabat definitions. Circles below the sequence are used to indicate residues which make main chain only contacts with VISTA, triangles indicate a side-chain contact, and squares indicate the side chain contact results in either a hydrogen bond or salt bridge interaction as calculated by PISA.

[0047] Figure 31: Comparison of epitope regions identified by crystallography and hydrogen deuterium exchange (HDX). Sequence of VISTA construct used in structure determination. Circles below the sequence are used to indicate residues which make only main chain contacts with VSTB112, triangles indicate a side chain contact, and squares indicate the side chain contact results in either a hydrogen bond or salt bridge interaction as calculated by PISA.

[0048] Figure 32: Activation of CD14+ monocytes in whole PBMC by VSTB174 (derived from VSTB112). In each part of the experiment, cells were incubated with PBS, IgG1 control antibody, or VSTB174 at 1, 0.1 or 0.01 ug/ml. Left panel shows CD80 MFI; right panel shows HLA-DR MFI (two donors tested with representative results shown).

[0049] Figure 33: Graph showing ADCC activity of VSTB174 directed against K562-VISTA cells.

[0050] Figure 34: Graph showing ADCP activity of VSTB174 directed against K562-VISTA cells. Both antibodies depicted have the same Fab, but VSTB174 has an IgG1 Fc and VSTB140 has Fc silent IgG2.

[0051] Figure 35: Graph showing phagocytosis mediated by VSTB174, VSTB149 or VSTB140 mAbs against K562-VISTA. Each mAb was tested with 7 half log doses, ranging from 0.0008μg/ml to 0.56ug/ml.

[0052] Figure 36: Graph showing phagocytosis mediated by VSTB174, VSTB149 or VSTB140 mAbs against myeloma cell line K562 cells. Each mAb was tested with 7 half log doses, ranging from 0.0008μg/ml to 0.56ug/ml.

[0053] Figure 37: MB49 tumor efficacy study evaluating VSTB123 1, 5, 7.5, and 10 mg/kg in female VISTA-KI mice. Tumor volumes were approximately 50 mm^3 when dosing began at day 6 after implant. VSTB123 is the VSTB112 Fab grafted onto a mouse Fc scaffold and binds to human VISTA in the VISTA-KI mouse.

[0054] Figure 38: Graph shows that CD14+ cells expressing high/intermediate levels of VISTA are found in 13/13 lung cancer samples, as well as in distant lung tissue and peripheral blood of patients.

[0055] Figure 39: IHC staining for VISTA in Lung Cancer using GG8.

[0056] Figure 40 is a graph of binding rate vs. antibody concentration.

[0057] Figure 41: Representative images of VISTA staining using VSTB175 (Figures 41A-41C) or negative control sera (Figure 41D) in lung cancer samples that have been formalin fixed and paraffin embedded. Figure 41A: small cell lung cancer; Figure 41B: small cell lung cancer; Figure 41C: squamous lung cancer; Figure 41D: small cell lung cancer.

[0058] Figure 42: VISTA (VSTB175) Staining in Human Tonsil. Shown are two regions tonsil tissue block stained with the VISTA (VSTB175) antibody (left and right panels).

[0059] Figure 43: VISTA (VSTB175) Staining Examples in Human Lung Cancer Tissues. Representative regions of three tissue samples are shown at 20X magnification and demonstrate VISTA staining observed across the study cohort.

[0060] Figure 44: Cell Line, VISTA-K562. Antibody VSTB175 showed frequent strong staining in VISTA-K562 cells. 40X magnification.

[0061] Figure 45: Cell Line, Raji. Antibody VSTB175 was negative in negative control Raji cells. 40X magnification.

[0062] Figure 46: Lung respiratory epithelium (left panel), alveoli (right panel). This sample of normal lung was obtained at surgery from a 43-year-old female. Antibody VSTB175 shows staining in intravascular leukocytes (mostly neutrophils) and in rare intraepithelial neutrophils within the respiratory epithelium. The epithelium itself and the remaining cell types, including macrophages, were negative. 40X magnification.

[0063] Figure 47: Lymph Node. This sample of normal lymph node was obtained from a 37-year-old male. Antibody VSTB175 was negative in lymph node tissue of the sinus. 40X magnification.

[0064] Figure 48: Placental villi. This sample of normal placenta was obtained at surgery from a 20-year-old female. Antibody VSTB175 showed membranous staining in trophoblasts. 40X magnification.

[0065] Figure 49: Spleen. This sample of normal spleen was obtained at autopsy from a 73-year-old female with Alzheimer's disease. Antibody VSTB175 showed focal staining in mononuclear cells, neutrophils, and sinusoidal lining cells in the red pulp. White pulp lymphocytes were mostly negative. 40X magnification.

[0066] Figure 50: Tonsil. This sample of normal tonsil was obtained from a 11-year-old female. Antibody VSTB175 showed focal staining in monocytes associated with the surface squamous epithelium, but most lymphocytes were negative. 40X magnification.

[0067] Figure 51: Colon, Carcinoma. This sample of colon was obtained at surgery from a 57-year-old female. Antibody VSTB175 was negative in this sample, including malignant cells and inflammatory cells (neutrophils). 40X magnification.

[0068] Figure 52: Melanoma. This sample of melanoma was obtained at surgery from a 54-year-old female. Antibody VSTB175 showed focal staining in benign macrophages but was negative in malignant cells. 40X magnification.

[0069] Figure 53: Ovary, Carcinoma. This sample of ovary was obtained from a 53-year-old female. Antibody VSTB175 showed focal cytoplasmic staining in intravascular leukocytes, including both mononuclear cells and polymorphonuclear neutrophils. Malignant cells were negative, as were rarely identified interspersed inflammatory cells. 40X magnification.

DETAILED DESCRIPTION OF THE INVENTION

[0070] A description of example embodiments of the invention follows.

[0071] The present invention relates to antibodies to novel Immunoglobulin family ligand designated V-domain Immunoglobulin Suppressor of T cell Activation (VISTA) (Genbank: JN602184) (Wang et al., 2010, 2011). VISTA bears homology to PD-L1 but displays a unique expression pattern that is restricted to the hematopoietic compartment. Specifically, VISTA is constitutively and highly expressed on CD11b^{high} myeloid cells, and expressed at lower levels on CD4$^+$ and CD8$^+$ T cells. The human homologue shares approximately 85% homology with murine VISTA and has similar expression patterns (Lines et al., Cancer Research 74:1924, 2014). VISTA expressed on antigen presenting cells (APCs) suppresses CD4$^+$ and CD8$^+$ T cell proliferation and cytokine production via a cognate receptor independent of PD-1. In a passive EAE (experimental autoimmune encephalomyelitis) disease model, a VISTA specific monoclonal antibody enhanced T-cell dependent immune responses and exacerbated disease. VISTA over-expression on tumor cells impaired protective anti-tumor immunity in tumor-bearing hosts. Studies of human VISTA confirmed its suppressive function on human T cells (Lines et al Cancer Research 74:1924, 2014,. Studies from Flies et al. also identified VISTA (named PD-1H) as a potent immune suppressive molecule (Flies et al., 2011). VISTA is described in further detail in U.S. Published application US 20130177557 A1 and U.S. Patent Nos. 7,919,585 and 8,236,304.

[0072] As described in Example 12 herein, treatment with a VISTA-specific monoclonal antibody in murine tumor models has been shown to reverse the suppressive character of the tumor immune microenvironment and enhance protective anti-tumor immunity, thus, demonstrating the potential of a VISTA monoclonal antibody as a novel therapeutic for cancer immunotherapy.

[0073] ANTIBODIES AND FRAGMENTS OF THE PRESENT INVENTION

[0074] The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, humanized antibodies, human antibodies and anti-idiotypic (anti-Id) antibodies, as well as fragments, regions or derivatives thereof, provided by any known technique, such as, but not limited to, enzymatic cleavage, peptide synthesis or recombinant techniques. Anti-VISTA antibodies of the present invention are capable of binding portions of VISTA that modulate, regulate, or enhance an immune response. In some embodiments, the antibodies competitively inhibit one or more of the anti-VISTA antibodies described herein. Methods for determining whether two or more antibodies compete for binding to the same target are known in the art. For example, a competitive binding assay can be used to determine whether one antibody blocks the binding of another antibody to the target. Typically, a competitive binding assay involves the use of purified target antigen (*e.g.*, PD-1) bound either to a solid substrate or cells, an unlabeled test binding molecule, and a labeled reference binding molecule. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test binding molecule. Usually the test binding molecule is present in excess. Typically, when a competing binding molecule is present in excess, it will inhibit specific binding of a reference binding molecule to a common antigen by at least 50-55%, 55-60%, 60-65%, 65-70%, 70-75%, or more. In some embodiments, competitive inhibition is determined using a competitive inhibition ELISA assay.

[0075] Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. Monoclonal antibodies may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, *Nature,* 256:495-497 (1975); U.S. Pat. No. 4,376,110; Ausubel et al., eds., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1987, 1992); and Harlow and Lane ANTIBODIES: A Laboratory Manual Cold Spring Harbor Laboratory (1988); Colligan et al., eds., Current Protocols in Immunology, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A hybridoma producing a monoclonal antibody of the present invention may be cultivated *in vitro, in situ* or *in vivo.*

[0076] The invention also encompasses digestion fragments, specified portions and variants thereof, including antibody mimetics or comprising portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. Functional fragments include antigen-binding fragments that bind to a mammalian VISTA protein. For example, antibody fragments capable of binding to VISTA or portions thereof, including, but not limited to Fab (*e.g.*, by papain digestion), Fab′ (*e.g.*, by pepsin digestion and partial reduction) and F(ab′)$_2$ (*e.g.*, by pepsin digestion), facb (*e.g.*, by plasmin digestion), pFc′ (*e.g.*, by pepsin or plasmin digestion), Fd (*e.g.*, by pepsin digestion, partial reduction and reaggregation), Fv or scFv (*e.g.*, by molecular biology techniques) fragments, are encompassed by the invention (*see, e.g.*, Colligan, Immunology, supra). Antibody fragments of the present invention also include those discussed and described in Aaron L. Nelson, mAbs 2:1, 77-83 (January/February 2010).

[0077] Such fragments can be produced, for example, by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a combination gene encoding a F(ab′)$_2$ heavy chain portion can be designed to include DNA sequences encoding the CH1 domain and/or hinge region of the heavy chain. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

[0078] In one embodiment, the amino acid sequence of an immunoglobulin chain, or portion thereof (*e.g.*, variable region, CDR) has about 70-100% identity (*e.g.*, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or any range or value therein) to the amino acid sequence of the corresponding variable sequence chain described herein. Preferably, 70-100% amino acid identity (*e.g.*, 85, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or any range or value therein) is determined using a suitable computer algorithm, as known in the art.

[0079] Examples of heavy chain and light chain variable regions sequences are provided herein.

[0080] The antibodies of the present invention, or specified variants thereof, can comprise any number of contiguous amino acid residues from an antibody of the present invention, wherein that number is selected from the group of integers consisting of from 10-100% of the number of contiguous residues in an anti-TNF antibody. Optionally, this subsequence of contiguous amino acids is at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250 or more amino acids in length, or any range or value therein. Further, the number of such subsequences can be any integer selected from the group consisting of from 1 to 20, such as at least 2, 3, 4, or 5.

[0081] As those of skill will appreciate, the present invention includes at least one biologically active antibody of the present invention. Biologically active antibodies have a specific activity at least 20%, 30%, or 40%, and preferably at least 50%, 60%, or 70%, and most preferably at least 80%, 90%, or 95%-100% of that of the native (non-synthetic), endogenous or related and known antibody. Methods of assaying and quantifying measures of enzymatic activity and substrate specificity are well known to those of skill in the art.

[0082] Substantial similarity refers to a compound having at least 85% (*e.g.*, at least 95%) identity and at least 85% (*e.g.*, at least 95%) of activity of the native (non-synthetic), endogenous or related and known antibody.

[0083] As used herein, the term "human antibody" refers to an antibody in which substantially every part of the protein (*e.g.*, CDR, framework, CL, CH domains (*e.g.*, CH1, CH2, CH3), hinge, (VL, VH)) is substantially non-immunogenic in humans, with only minor sequence changes or variations. Similarly, antibodies designated primate (monkey, baboon, chimpanzee, and the like), rodent (mouse, rat, and the like) and other mammals designate such species, sub-genus, genus, sub-family, family specific antibodies. Further, chimeric antibodies can include any combination of the above. Such changes or variations optionally and preferably retain or reduce the immunogenicity in humans or other species relative to non-modified antibodies. Thus, a human antibody is distinct from a chimeric or humanized antibody. It is pointed out that a human antibody can be produced by a non-human animal or prokaryotic or eukaryotic cell that is capable of expressing functionally rearranged human immunoglobulin (*e.g.*, heavy chain and/or light chain) genes. Further, when a human antibody is a single chain antibody, it can comprise a linker peptide that is not found in native human antibodies. For example, an Fv can comprise a linker peptide, such as two to about eight glycine or other amino acid residues, which connects the variable region of the heavy chain and the variable region of the light chain. Such linker peptides are considered to be of human origin.

[0084] Bispecific, heterospecific, heteroconjugate or similar antibodies can also be used that are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for at least one VISTA protein, the other one is for any other antigen. Methods for making bispecific antibodies are known in the art. The recombinant production of bispecific antibodies can be based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature 305:537 (1983)). See also WO 93/08829, U.S. Pat. Nos. 6,210,668, 6,193,967, 6,132,992, 6,106,833, 6,060,285, 6,037,453, 6,010,902, 5,989,530, 5,959,084, 5,959,083, 5,932,448, 5,833,985, 5,821,333, 5,807,706, 5,643,759, 5,601,819, 5,582,996, 5,496,549, 4,676,980, WO 91/00360, WO 92/00373, EP 03089, Traunecker et al., EMBO J. 10:3655 (1991), Suresh et al., Methods in Enzymology 121:210 (1986).

[0085] In one embodiment, the invention relates to a bispecific antibody targeting VISTA and a second target protein (*e.g.*, an immune checkpoint protein). Exemplary bispecific anitbodies include a bispecific antibody targeting VISTA and PD-L1 and a bispecific antibody targeting VISTA and PD-L2.

[0086] Human antibodies that are specific for human VISTA proteins or fragments thereof can be raised against an appropriate immunogenic antigen, such as VISTA protein or a portion thereof (including synthetic molecules, such as synthetic peptides).

[0087] Other specific or general mammalian antibodies can be similarly raised. Immunogenic antigens preparation and monoclonal antibody production can be performed using any suitable technique.

[0088] For example, a hybridoma is produced by fusing a suitable immortal cell line (*e.g.*, a myeloma cell line such as, but not limited to, Sp2/0, Sp2/0-AG14, NSO, NS1, NS2, AE-1, L.5, >243, P3X63Ag8.653, Sp2 SA3, Sp2 MAI, Sp2 SS1, Sp2 SA5, U937, MLA 144, ACT IV, MOLT4, DA-1, JURKAT, WEHI, K-562, COS, RAJI, NIH 3T3, HL-60, MLA 144, NAMAIWA, NEURO 2A, or the like, or heteromylomas, fusion products thereof, or any cell or fusion cell derived therefrom, or any other suitable cell line as known in the art, See, *e.g.*, www.atcc.org, with antibody-producing cells. Antibody-producing cells can include isolated or cloned spleen, peripheral blood, lymph, tonsil, or other immune cells (*e.g.*, B cells), or any other cells expressing heavy or light chain constant or variable or framework or complementarity determining region (CDR) sequences. Such antibody-producing cells can be recombinant or endogenous cells, and can also be prokaryotic or eukaryotic (*e.g.*, mammalian, such as, rodent, equine, ovine, goat, sheep, primate),. See, *e.g.*, Ausubel, supra, and Colligan, Immunology, supra, chapter 2.

[0089] Antibody producing cells can also be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals that have been immunized with the antigen of interest. Any other suitable host cell can also be used for expressing heterologous or endogenous nucleic acid encoding an antibody, specified fragment or variant thereof, of the present invention. Fused cells (hybridomas) or recombinant cells can be isolated using selective culture conditions or other suitable known methods, and cloned by limiting dilution or cell sorting, or other known methods. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (*e.g.*, enzyme-linked immunosorbent assay (ELISA)).

[0090] Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (*e.g.*, but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, or the like, display library; *e.g.*, as available from Cambridge antibody Technologies, Cambridgeshire, UK; MorphoSys, Martinsreid/Planegg, DE; Biovation, Aberdeen, Scotland, UK; Bioinvent, Lund, Sweden; Dyax Corp., Enzon, Affymax/Biosite; Xoma, Berkeley, Calif.; Ixsys. See, *e.g.*, PCT/GB91/01134; PCT/GB92/01755; PCT/GB92/002240; PCT/GB92/00883; PCT/GB93/00605; PCT/GB94/01422; PCT/GB94/02662; PCT/GB97/01835; ; WO90/14443; WO90/14424; WO90/14430; PCT/U594/1234; WO92/18619; WO96/07754; EP 614 989 ; WO95/16027 ; WO88/06630; WO90/3809 ; U.S. Pat. No. 4,704,692 ; PCT/US91/02989 ; WO89/06283; EP 371 998; EP 550 400; ; EP 229 046; PCT/US91/07149 ; or stochastically-generated peptides or proteins--U.S. Patent Nos. 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862;WO 86/05803, EP 590 689, or that rely upon immunization of transgenic animals (*e.g.*, SCID mice, Nguyen et al., Microbiol. Immunol. 41:901-907 (1997); Sandhu et al., Crit. Rev. Biotechnol. 16:95-118 (1996); Eren et al., Immunol. 93:154-161 (1998), as well as related patents and applications) that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes et al., Proc. Natl. Acad. Sci. USA, 94:4937-4942 (May 1997); Hanes et al., Proc. Natl. Acad. Sci. USA, 95:14130-14135 (November 1998)); single cell antibody producing technologies (U.S. Pat. No. 5,627,052, Wen et al., J. Immunol. 17:887-892 (1987); Babcook et al., Proc. Natl. Acad. Sci. USA 93:7843-7848 (1996)); gel microdroplet and flow cytometry (Powell et al., Biotechnol. 8:333-337 (1990); One Cell Systems, Cambridge, Mass.; Gray et al., J. Imm. Meth. 182:155-163 (1995); Kenny et al., Bio/Technol. 13:787-790 (1995)); B-cell selection (Steenbakkers et al., Molec. Biol. Reports 19:125-134 (1994); Jonak et al., Progress Biotech, Vol. 5, In Vitro Immunization in Hybridoma Technology, Borrebaeck, ed., Elsevier Science Publishers B.V., Amsterdam, Netherlands (1988)).

[0091] Methods for engineering or humanizing non-human or human antibodies can also be used and are well known in the art. Generally, a humanized or engineered antibody has one or more amino acid residues from a source which is non-human, *e.g.*, but not limited to mouse, rat, rabbit, non-human primate or other mammal. These human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable, constant or other domain of a known human sequence. Known human Ig sequences are disclosed, *e.g.*, www.ncbi.nlm.nih.gov/entrez/query.fcgi; www.atcc.org/phage/hdb.html.

[0092] Such imported sequences can be used to reduce immunogenicity or reduce, enhance or modify binding, affinity, avidity, specificity, half-life, or any other suitable characteristic, as known in the art. Generally part or all of the non-human or human CDR sequences are maintained while part or all of the non-human sequences of the framework and/or constant regions are replaced with human or other amino acids. Antibodies can also optionally be humanized with retention of high affinity for the antigen and other favorable biological properties using three-dimensional immunoglobulin models that are known to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, framework (FR) residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to those described in, for example, Winter (Jones et al., Nature 321:522 (1986); Riechmann et al., Nature 332:323 (1988); Verhoeyen et al., Science 239:1534 (1988)), Sims et al., J. Immunol. 151: 2296 (1993); Chothia and Lesk, J. Mol. Biol. 196:901 (1987), Carter et al., Proc. Natl. Acad. Sci. U.S.A. 89:4285 (1992); Presta et al., J. Immunol. 151:2623 (1993), U.S. Pat. Nos. 5,723,323, 5,976862, 5,824514, 5,817483, 5,814476, 5,763,192, 5,723,323, 5,766,886, 5,714,352, 6,204,023, 6,180,370, 5,693,762, 5,530,101, 5,585,089, 5,225,539; 4,816,567included references cited therein.

[0093] The anti-VISTA antibody can also be optionally generated by immunization of a transgenic animal (*e.g.*, mouse, rat, rabbit, hamster, non-human primate, and the like) capable of producing a repertoire of human antibodies, as described herein and/or as known in the art. Cells that produce a human anti-VISTA antibody can be isolated from such animals and immortalized using suitable methods, such as the methods described herein.

[0094] Transgenic animals that can produce a repertoire of human antibodies that bind to human antigens can be produced by known methods (*e.g.*, but not limited to, U.S. Pat. Nos. 5,770,428, 5,569,825, 5,545,806, 5,625,126, 5,625,825, 5,633,425, 5,661,016 and 5,789,650 issued to Lonberg et al.; Jakobovits et al. WO 98/50433, Jakobovits et al. WO 98/24893, Lonberg et al. WO 98/24884, Lonberg et al. WO 97/13852, Lonberg et al. WO 94/25585, Kucherlapate et al. WO 96/34096, Kucherlapate et al. EP 0463 151 B1, Kucherlapate et al. EP 0710 719 A1, Surani et al. U.S. Pat. No. 5,545,807, Bruggemann et al. WO 90/04036, Bruggemann et al. EP 0438 474 B1, Lonberg et al. EP 0814 259 A2, Lonberg et al. GB 2 272 440 A, Lonberg et al. Nature 368:856-859 (1994), Taylor et al., Int. Immunol. 6(4)579-591 (1994), Green et al, Nature Genetics 7:13-21 (1994), Mendez et al., Nature Genetics 15:146-156 (1997), Taylor et al., Nucleic Acids Research 20(23):6287-6295 (1992), Tuaillon et al., Proc Natl Acad Sci USA 90(8)3720-3724 (1993), Lonberg et al., Int Rev Immunol 13(1):65-93 (1995) and Fishwald et al., Nat Biotechnol 14(7):845-851 (1996)). Generally, these mice comprise at least one transgene comprising DNA from at least one human immunoglobulin locus that is functionally rearranged, or which can undergo functional rearrangement. The endogenous immunoglobulin loci in such mice can be disrupted or deleted to eliminate the capacity of the animal to produce antibodies encoded by endogenous genes.

[0095] Screening antibodies for specific binding to similar proteins or fragments can be conveniently achieved using peptide display libraries. This method involves the screening of large collections of peptides for individual members having the desired function or structure.

Antibody screening of peptide display libraries is well known in the art. The displayed peptide sequences can be from 3 to 5000 or more amino acids in length, frequently from 5-100 amino acids long, and often from about 8 to 25 amino acids long. In addition to direct chemical synthetic methods for generating peptide libraries, several recombinant DNA methods have been described. One type involves the display of a peptide sequence on the surface of a bacteriophage or cell. Each bacteriophage or cell contains the nucleotide sequence encoding the particular displayed peptide sequence. Such methods are described in PCT Patent Publication Nos. 91/17271, 91/18980, 91/19818, and 93/08278. Other systems for generating libraries of peptides have aspects of both in vitro chemical synthesis and recombinant methods. See, PCT Patent Publication Nos. 92/05258, 92/14843, and 96/19256. See also, U.S. Patent Nos. 5,658,754; and 5,643,768. Peptide display libraries, vector, and screening kits are commercially available from such suppliers as Invitrogen (Carlsbad, Calif.), and Cambridge antibody Technologies (Cambridgeshire, UK). See, *e.g.*, U.S. Patent Nos. 4,704,692, 4,939,666, 4,946,778, 5,260,203, 5,455,030, 5,518,889, 5,534,621, 5,656,730, 5,763,733, 5,767,260, 5,856,456; 5,223,409, 5,403,484, 5,571,698, 5,837,500, assigned to Dyax, 5,427,908, 5,580,717; 5,885,793, assigned to Cambridge antibody Technologies; 5,750,373, assigned to Genentech, 5,618,920, 5,595,898, 5,576,195, 5,698,435, 5,693,493, and 5,698,417.

[0096] Antibodies of the present invention can also be prepared using at least one anti-VISTA antibody encoding nucleic acid to provide transgenic animals, such as goats, cows, sheep, and the like, that produce such antibodies in their milk. Such animals can be provided using known methods. See, *e.g.*, but not limited to, U.S. Pat. Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; 5,304,489, and the like.

[0097] The anti-VISTA antibodies of the present invention can also be produced using transgenic plants, according to known methods. See also, *e.g.*, Fischer et al., Biotechnol. Appl. Biochem. 30:99-108 (October, 1999), Cramer et al., Curr. Top. Microbol. Immunol. 240:95-118 (1999) and references cited therein; Ma et al., Trends Biotechnol. 13:522-7 (1995); Ma et al., Plant Physiol. 109:341-6 (1995); Whitelam et al., Biochem. Soc. Trans. 22:940-944 (1994); and references cited therein.

[0098] The antibodies of the invention can bind human VISTA with a wide range of affinities (K_D). In a preferred embodiment, at least one human monoclonal antibody of the present invention can optionally bind human VISTA with high affinity. For example, a human monoclonal antibody can bind human VISTA with a K_D equal to or less than about 10^{-7} M, such as but not limited to, 0.1-9.9 (or any range or value therein) x 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13} or any range or value therein. In some embodiments, the antibody or antibody fragment can binds human VISTA with an affinity of at least $1x10^{-7}$ liter/mole, for example, at least $1x10^{-8}$ liter/mole, for example, at least $1x10^{-9}$ liter/mole liter/mole.

[0099] The affinity or avidity of an antibody for an antigen can be determined experimentally using any suitable method. (See, for example, Berzofsky, et al., "Antibody-Antigen Interactions," In Fundamental Immunology, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, Janis Immunology, W.H. Freeman and Company: New York, N.Y. (1992); and methods described herein). The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions (*e.g.*, salt concentration, pH). Thus, measurements of affinity and other antigen-binding parameters (*e.g.*, K_D, K_a, K_d) are preferably made with standardized solutions of antibody and antigen, and a standardized buffer.

[00100] NUCLEIC ACID MOLECULES

[00101] Using the information provided herein, such as the nucleotide sequences encoding at least 70-100% of the contiguous amino acids of at least one of specified fragments, variants or consensus sequences thereof, or a deposited vector comprising at least one of these sequences, a nucleic acid molecule of the present invention encoding at least one anti-VISTA antibody comprising all of the heavy chain variable CDR regions of SEQ ID NOS:1, 2 and 3 and/or all of the light chain variable CDR regions of SEQ ID NOS:4, 5 and 6 can be obtained using methods described herein or as known in the art.

[00102] Nucleic acid molecules of the present invention can be in the form of RNA, such as mRNA, hnRNA, tRNA or any other form, or in the form of DNA, including, but not limited to, cDNA and genomic DNA obtained by cloning or produced synthetically, or any combinations thereof. The DNA can be triple-stranded, double-stranded or single-stranded, or any combination thereof. Any portion of at least one strand of the DNA or RNA can be the coding strand, also known as the sense strand, or it can be the non-coding strand, also referred to as the anti-sense strand.

[00103] Isolated nucleic acid molecules of the present invention can include nucleic acid molecules comprising an open reading frame (ORF), for example, but not limited to, at least one specified portion of at least one CDR, as CDR1, CDR2 and/or CDR3 of at least one heavy chain or light chain; nucleic acid molecules comprising the coding sequence for an anti-VISTA antibody or fragment, *e.g.*, a fragment comprising a variable region; and nucleic acid molecules which comprise a nucleotide sequence different from those described above but which, due to the degeneracy of the genetic code, still encode at least one anti-VISTA antibody as described herein and/or as known in the art. It would be routine for one skilled in the art to generate such degenerate nucleic acid variants that code for specific anti-VISTA antibodies of the present invention. See, *e.g.*, Ausubel, et al., supra, and such nucleic acid variants are included in the present invention.

[00104] As indicated herein, nucleic acid molecules of the present invention which comprise a nucleic acid encoding an anti-VISTA antibody can include, but are not limited to, those encoding the amino acid sequence of an antibody fragment; the coding sequence for the entire antibody or a portion thereof; the coding sequence for an antibody, fragment or portion, as well as additional sequences, such as the coding sequence of at least one signal leader or fusion peptide, with or without the aforementioned additional coding sequences, such as at least one intron, together with additional, non-coding sequences, including but not limited to, non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals (for example--ribosome binding and stability of mRNA); an additional coding sequence that codes for additional amino acids, such as those that provide additional functionalities. Thus, the sequence encoding an antibody can be fused to a marker sequence, such as a sequence encoding a peptide that facilitates purification of the fused antibody comprising an antibody fragment or portion.

[00105] Human genes which encode the constant (C) regions of the antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C regions genes can be derived from any human cell including those which express and produce human immunoglobulins. The human C_H region can be derived from any of the known classes or isotypes of human H chains, including γ, μ, α, δ or ε and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of an antibody, the choice of C_H region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC).

[00106] COMPOSITIONS

[00107] The pharmaceutical compositions disclosed herein are prepared in accordance with standard procedures and are administered at dosages that are selected to treat, *e.g.*, reduce, prevent, or eliminate, or to slow or halt the progression of, the condition being treated (See, *e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, and Goodman and Gilman's The Pharmaceutical Basis of Therapeutics, McGraw-Hill, New York, N.Y. for a general description of the methods for administering various agents for human therapy). The compositions comprising the disclosed antibodies and agents can be delivered using controlled or sustained-release delivery systems (*e.g.*, capsules, biodegradable matrices). Examples of delayed-release delivery systems for drug delivery that would be suitable for administration of the compositions of the disclosed compounds are described in, *e.g.*, U.S. Patent Nos. US 5,990,092; 5,039,660; 4,452,775; and 3,854,480

[00108] For preparing pharmaceutical compositions from the anti-VISTA antibodies and/or fragments of the present invention, pharmaceutically acceptable carriers can be solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. For example, the compounds of the present invention can be in powder form for reconstitution at the time of delivery. A solid carrier can be one or more substances which can also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. In powders, the carrier is a finely divided solid which is in a mixture with the finely divided active ingredient.

[00109] The powders and tablets preferably contain from about one to about seventy percent of the active ingredient. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium caboxymethylcellulose, a low-melting wax, cocoa butter, and the like. Tablets, powders, cachets, lozenges, fast-melt strips, capsules and pills can be used as solid dosage forms containing the active ingredient suitable for oral administration.

[00110] Liquid form preparations include solutions, suspensions, retention enemas, and emulsions, for example, water or water propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

[00111] The pharmaceutical composition can be in unit dosage form. In such form, the composition is subdivided into unit doses containing appropriate quantities of the active ingredient. The unit dosage form can be a packaged preparation, the package containing discrete quantities of unit doses. The dosages can be varied depending upon the requirements of the patient, the severity of the condition being treated, the compound and the route of administration being employed. Determination of the proper dosage for a particular situation is within the skill in the art.

[00112] Also, the pharmaceutical composition can contain, if desired, other compatible agents, *e.g.*, pharmaceutical, therapeutic or prophylactic agents. Therapeutic or prophylactic agents include, but are not limited to, peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules. Examples of the classes of such agents (*e.g.*, anti-cancer agents) include, but are not limited to, cytotoxins, angiogenesis inhibitors, immunomodulatory agents, immuno-oncology agents, and agents used to provide relief from pain or to offset the deleterious effects of one or more therapeutic agents (*e.g.*, bisphosphonate use to reduce the hypercalcemic effects of glucocorticoids).

[00113] Angiogenesis inhibitors, agents and therapies that are suitable for use in the compositions and methods described herein include, but are not limited to, angiostatin (plasminogen fragment); antiangiogenic antithrombin III; angiozyme. Bisphosphonates include, but are not limited to, alendronate, clodronate, etidronate, ibandronate, pamidronate, risedronate, tiludronate, and zoledronate.

[00114] Immunomodulatory agents and therapies that are suitable for use in the compositions and methods described herein include, but are not limited to, anti-T cell receptor antibodies such as anti-CD3 antibodies (*e.g.* Nuvion (Protein Design Labs), OKT3 (Johnson & Johnson), or anti-CD20 antibodies Rituxan (IDEC)), anti-CD52 antibodies (*e.g.* CAMPATH 1H (Ilex)), anti-CD11a antibodies (*e.g.* Xanelim (Genentech)); anti-cytokine or anti-cytokine receptor antibodies and antagonists such as anti-IL-2 receptor antibodies (Zenapax (Protein Design Labs)), anti-IL-6 receptor antibodies (*e.g.* MRA (Chugai)), and anti-IL-12 antibodies (CNTO1275(Janssen)), anti-TNFalpha antibodies (Remicade(Janssen)) or TNF receptor antagonist (Enbrel (Immunex)), anti-IL-6 antibodies (BE8 (Diaclone) and siltuximab (CNTO32 (Centocor)), and antibodies that immunospecifically bind to tumor-associated antigens (*e.g.*, trastuzimab (Genentech)).

[00115] Immuno-oncology agents that are suitable for use in the compositions and methods described herein include, but are not limited to, ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), anti-PD-L1 antibodies, and anti-LAG-3 antibodies.

[00116] The composition is preferably made in the form of a dosage unit containing a therapeutically effective amount of the antibody or fragment. Examples of dosage units are tablets and capsules. For therapeutic purposes, the tablets and capsules can contain, in addition to the active ingredient, conventional carriers such as binding agents, for example, acacia gum, gelatin, polyvinylpyrrolidone, sorbitol, or tragacanth; fillers, for example, calcium phosphate, glycine, lactose, maize-starch, sorbitol, or sucrose; lubricants, for example, magnesium stearate, polyethylene glycol, silica, or talc; disintegrants, for example potato starch, flavoring or coloring agents, or acceptable wetting agents. Oral liquid preparations generally in the form of aqueous or oily solutions, suspensions, emulsions, syrups or elixirs can contain conventional additives such as suspending agents, emulsifying agents, non-aqueous agents, preservatives, coloring agents and flavoring agents. Examples of additives for liquid preparations include acacia, almond oil, ethyl alcohol, fractionated coconut oil, gelatin, glucose syrup, glycerin, hydrogenated edible fats, lecithin, methyl cellulose, methyl or propyl para-hydroxybenzoate, propylene glycol, sorbitol, or sorbic acid.

[00117] Other general details regarding methods of making and using the compounds and compositions described herein are well-known in the art. See, *e.g.*, U.S. Patent No. 7,820,169.

[00118] METHODS OF TREATMENT

[00119] One of skill in the art, *e.g.*, a clinician, can determine the suitable dosage and route of administration for a particular antibody, fragment or composition for administration to an individual, considering the agents chosen, pharmaceutical formulation and route of administration, various patient factors and other considerations. Preferably, the dosage does not cause or produces minimal or no adverse side effects. In standard multi-dosing regimens, a pharmacological agent may be administered on a dosage schedule that is designed to maintain a pre-determined or optimal plasma concentration in the subject undergoing treatment. The antibodies, fragments and compositions can be added at any appropriate dosage ranges or therapeutically effective amount, for example, 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1.0 mg/kg, 1.5 mg/kg, 2.0 mg/kg, 2.5 mg/kg, 3.0 mg/kg, 4.0 mg/kg, 5.0 mg/kg, 6.0 mg/kg, 7.0 mg/kg, 8.0 mg/kg, 9.0 mg/kg, 10.0 mg/kg, 11.0 mg/kg, 12.0 mg/kg, 13.0 mg/kg, 14.0 mg/kg, 15.0 mg/kg, 16.0 mg/kg, 17.0 mg/kg, 18.0 mg/kg, 19.0 mg/kg, 20.0 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg 60 mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg and 100 mg/kg. In one embodiment, the dosage of the administered composition, antibody or fragment is 0.1-15 mg/kg per administration.

[00120] The antibody or fragment can be administered once, at least once, twice, at least twice, three times, or at least three times per day. The antibody or fragment can be administered once, at least once, twice, at least twice, three times, at least three times, four times, at least four times, five times, at least five times, six times per week, or at least six times per week. The antibody or fragment can be administered once per month, at least once per month, twice per month, at least twice per month, three times per month or at least three times per month. The antibody or antibody fragment can be administered once per year, at least once per year, twice per year, at least twice per year, three times per year, at least three times per year, four times per year, at least four times per year, five times per year, at least five times per year, six times per year or at least six times per year.

[00121] The anti-VISTA antibodies, fragments and compositions can, for example, be administered through parenteral or nonparenteral means, including, but not limited to, intravenously, subcutaneously, orally, rectally, intramuscularly, intraperitoneally, transmucosally, transdermally, intrathecally, nasally, or topically. One of ordinary skill in the art will recognize that the following dosage forms can comprise as the active ingredient, either compounds or a corresponding pharmaceutically acceptable salt of a compound of the present invention. In some embodiments, the dosage forms can comprise as the active ingredient, either a compound or a corresponding pharmaceutically acceptable salt of a compound.

[00122] The anti-VISTA antibodies of the invention can be administered as part of a combination therapy (*e.g.*, with each other, or with one or more other therapeutic agents). The compounds of the invention can be administered before, after or concurrently with one or more other therapeutic agents. In some embodiments, a compound of the invention and other therapeutic agent can be co-administered simultaneously (*e.g.*, concurrently) as either separate formulations or as a joint formulation. Alternatively, the agents can be administered sequentially, as separate compositions, within an appropriate time frame, as determined by the skilled clinician (*e.g.*, a time sufficient to allow an overlap of the pharmaceutical effects of the therapies). A compound of the invention and one or more other therapeutic agents can be administered in a single dose or in multiple doses, in an order and on a schedule suitable to achieve a desired therapeutic effect.

[00123] The present invention also provides a method for modulating or treating at least one malignant disease in a cell, tissue, organ, animal or patient. In some embodiments, the compounds and compositions of the present invention are used to treat or prevent cancer. Cancer can include any malignant or benign tumor of any organ or body system. Examples include, but are not limited to, the following: breast, digestive/gastrointestinal, endocrine, neuroendocrine, eye, genitourinary, germ cell, gynecologic, head and neck, hematologic/blood, musculoskeletal, neurologic, respiratory/thoracic, bladder, colon, rectal, lung, endometrial, kidney, pancreatic, liver, stomach, testicular, esophageal, prostate, brain, cervical, ovarian and thyroid cancers. Other cancers can include leukemias, melanomas, and lymphomas, and any cancer described herein. In some embodiments, the solid tumor is infiltrated with myeloid and/or T-cells. In some embodiments, the cancer is a leukemia, lymphoma, myelodysplastic syndrome and/or myeloma. In some embodiments, the cancer can be any kind or type of leukemia, including a lymphocytic leukemia or a myelogenous leukemia, such as, *e.g.*, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid (myelogenous) leukemia (AML), chronic myelogenous leukemia (CML), hairy cell leukemia, T-cell prolymphocytic leukemia, large granular lymphocytic leukemia, or adult T-cell leukemia. In some embodiments, the lymphoma is a histocytic lymphoma, follicular lymphoma or Hodgkin lymphoma, and in some embodiments, the cancer is a multiple myeloma. In some embodiments, the cancer is a solid tumor, for example, a melanoma, or bladder cancer. In a particular embodiment, the cancer is a lung cancer, such as a non-small cell lung cancer (NSCLC).

[00124] The present invention also provides a method for modulating or treating at least one malignant disease in a cell, tissue, organ, animal or patient, including, but not limited to, at least one of: leukemia, acute leukemia, acute lymphoblastic leukemia (ALL), B-cell, T-cell or FAB ALL, acute myeloid leukemia (AML), chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), hairy cell leukemia, myelodysplastic syndrome (MDS), a lymphoma, Hodgkin's disease, a malignant lymphoma, non-hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, Kaposi's sarcoma, colorectal carcinoma, pancreatic carcinoma, nasopharyngeal carcinoma, malignant histiocytosis, paraneoplastic syndrome/hypercalcemia of malignancy, solid tumors, adenocarcinomas, sarcomas, malignant melanoma, hemangioma, metastatic disease, cancer related bone resorption, cancer-related bone pain, and the like. In some embodiments, the solid tumor is infiltrated with myeloid and/or T-cells. In a particular embodiment, the solid tumor is a lung cancer, such as a non-small cell lung cancer (NSCLC).

[00125] In some embodiments, the compounds and therapies described herein are co-administered with a vaccine (such as a viral vector vaccine, bacterial vaccine, cell-based vaccine, DNA vaccine, RNA vaccine, peptide vaccine, or protein vaccine). Such vaccines are well known in the art. *See, e.g.,* Jeffrey Schlom, "Therapeutic Cancer Vaccines: Current Status and Moving Forward," *J Natl Cancer Inst*; 104:599–613 (2012).

[00126] In some embodiments, the compounds and therapies described herein are co-administered with agents for chemotherapy, hormone therapies and biological therapies, and/or bisphosphonates. In some embodiments, the agent(s) for chemotherapy include one or more of the following: arboplatin (Paraplatin), cisplatin (Platinol, Platinol-AQ), cyclophosphamide (Cytoxan, Neosar), doxorubicin (Adriamycin), etoposide (VePesid), fluorouracil (5-FU), gemcitabine (Gemzar), irinotecan (Camptosar), paclitaxel (Taxol), topotecan (Hycamtin), vincristine (Oncovin, Vincasar PFS), vinblastine (Velban).

[00127] In other embodiments, the anti-VISTA compounds and therapies described herein are co-administered with one or more immune checkpoint antibodies, such as, for example, nivolumab, pembrolizumab, tremelimumab, ipilimumab, anti-PD-L1 antibody, anti-PD-L2 antibody, anti-TIM-3 antibody, anti-LAG-3v, anti-OX40 antibody and anti-GITR antibody.

[00128] In another embodiment, the anti-VISTA compounds and therapies described herein are co-administered with a small molecule inhibitor of indoleamine 2,3-dioxygenase (IDO).

[00129] The anti-VISTA compounds and composition of the invention may be administered to a subject in need thereof to prevent (including preventing the recurrence of cancer) or treat (*e.g.*, manage or ameliorate a cancer or one or more symptoms thereof) cancer. Any agent or therapy (*e.g.*, chemotherapies, radiation therapies, targeted therapies, such as imatinib, sorafenib and vemurafenib, hormonal therapies, and/or biological therapies or immunotherapies) which is known to be useful, or which has been used or is currently being used for the prevention, treatment, management or amelioration of cancer or one or more symptoms thereof can be used in combination with a compound or composition of the invention described herein. Anti-cancer agents, but not limited to: 5-fluoruracil; acivicin; aldesleukin; altretamine; aminoglutethimide; amsacrine; anastrozole; anthramycin; asparaginase; azacitidine; azetepa; azotomycin; batimastat; bicalutamide; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflornithine hydrochloride; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; interleukin II (including recombinant interleukin II, or rIL2) , interferon alpha-2a; interferon alpha-2b; interferon alpha-m; interferon alpha-n3; interferon beta-I a; interferon gamma-I b; iproplatin; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; ormaplatin; paclitaxel; pegaspargase; porfromycin; prednimustine; procarbazine hydrochloride; puromycin; rogletimide; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; topotecan; trimetrexate; trimetrexate glucuronate; triptorelin; uracil mustard; uredepa; vapreotide; verteporfn; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; zorubicin hydrochloride. Targeted therapies include, but are not limited to, tyrosine kinase inhibitors (*e.g.*, imatinib, sorafenib, and vemurafenib). The invention also encompasses administration of an anti-VISTA compound of the invention in combination with radiation therapy comprising the use of x-rays, gamma rays and other sources of radiation to destroy the cancer cells. Cancer treatments are known in the art and have been described in such literature as the Physician's Desk Reference (57th ed., 2003).

[00130] The anti-VISTA antibodies described herein are also useful, for example, in the treatment of chronic infectious diseases, such as HIV, HBV, HCV, and HSV, among others.

[00131] DETECTION OF VISTA PROTEIN

[00132] The antibodies and antigen-binding fragments of the invention described herein are generally useful for, *e.g.*, detecting VISTA protein in a sample (*e.g.*, a biological material). The antibodies of the invention are compatible with many protein detection assays that are known to those of skill in the art, such as, for example, immunological and immunochemical methods including, but not limited to, flow cytometry (*e.g.*, FACS analysis), enzyme-linked immunosorbent assays (ELISA), including chemiluminescence assays, radioimmunoassay, immunoblot (*e.g.*, Western blot), immunohistochemistry (IHC), immunoprecipitation and other antibody-based quantitative methods (*e.g.*, Luminex® beads-based assays). Other suitable methods include, for example, mass spectroscopy.

[00133] The VISTA detection methods of the invention generally comprise contacting a sample (*e.g.*, a cell sample) with an anti-VISTA antibody or antigen-binding fragment of the inveniton described herein under conditions in which the antibody or antigen-binding fragment binds to VISTA protein in the sample. Suitable conditions for binding of an antibody or antigen-binding fragment of the invention to VISTA protein in a smaple can be readily determined by those of ordinary skill in the art and include a variety of conditions known in the art. Such conditions include, for example, those described in Example 27 herein.

[00134] For example, antibodies to VISTA can be used to determine the presence and/or expression level of VISTA in a sample directly or indirectly using, *e.g.*, immunohistochemistry (IHC). IHC specifically provides a method of detecting targets in a sample or tissue specimen *in situ* (see Mokry 1996, ACTA MEDICA 39:129). The overall cellular integrity of the sample is maintained in IHC, thus allowing detection of both the presence and location of the targets of interest.

[00135] An IHC staining procedure can comprise steps such as: cutting and trimming tissue, fixation, dehydration, paraffin infiltration, cutting in thin sections, mounting onto glass slides, baking, deparaffinization, rehydration, antigen retrieval, blocking steps, applying primary antibody, washing, applying secondary antibody-enzyme conjugate, washing, applying a tertiary antibody conjugated to a polymer and linked with an enzyme, applying a chromogen substrate, washing, counter staining, applying a cover slip and microscopic examination. Washing steps may be performed with any suitable buffer or solvent, *e.g.*, phosphate-buffered saline, TRIS-buffered saline, distilled water. The wash buffer may optionally contain a detergent, *e.g.*, TWEEN®-20 or NP-40.

[00136] In one embodiment, an IHC sample is fixed with formalin, embedded in paraffin and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labeling or secondary antibody-based or hapten-based labeling. Examples of known IHC systems include, for example, EnVision™(DakoCytomation), Powervision® (Immunovision, Springdale, Ariz.), the NBA® kit (Zymed Laboratories Inc., South San Francisco, Calif.), HistoFine® (Nichirei Corp, Tokyo, Japan).

[00137] VISTA protein can be detected in a variety of sample types, including but not limited to, a tissue sample, a biological fluid sample (*e.g.* mammalian plasma, serum, lymph, whole blood, spinal, amniotic, or other animal-derived fluid), a cell(s) (*e.g.*, a tumor cell, an immune cell) sample, and the like. Samples can include, for instance: (a) preparations comprising un-fixed fresh tissues and/or cells; (b) fixed and embedded tissue specimens, such as archived material; and (c) frozen tissues or cells. Thus, samples can be fresh or preserved, for example, in liquid solution, flash-frozen or lyophilized, smeared or dried, embedded, or fixed on slides or other supports.

[00138] In some embodiments, tissue or cell samples are fixed or embedded. Fixatives are used, for example, to preserve cells and tissues in a reproducible and life-like manner. Fixatives also stabilize cells and tissues, thereby protecting them from the rigors of processing and staining techniques. For example, samples comprising tissue blocks, sections, or smears can be immersed in a fixative fluid, or in the case of smears, dried.

[00139] Many methods of fixing and embedding tissue specimens are known, for example, alcohol fixation and formalin-fixation and subsequent paraffin embedding (FFPE). Any suitable fixing agent may be used. Examples include ethanol, acetic acid, picric acid, 2-propanol, 3,3'-diaminobenzidine tetrahydrochloride dihydrate, acetoin (mixture of monomer) and dimer, acrolein, crotonaldehyde (cis+trans), formaldehyde, glutaraldehyde, glyoxal, potassium dichromate, potassium permanganate, osmium tetroxide, paraformaldehyde, mercuric chloride, tolylene-2,4-diisocyanate, trichloroacetic acid, tungstic acid. Other examples include formalin (aqueous formaldehyde) and neutral buffered formalin, glutaraldehyde, carbodiimide, imidates, benzoequinone, osmic acid and osmium tetraoxide. Fresh biopsy specimens, cytological preparations (including touch preparations and blood smears), frozen sections, and tissues for IHC analysis may be fixed in organic solvents, including ethanol, acetic acid, methanol and/or acetone.

[00140] Any means of sampling from a subject, for example, by blood draw, spinal tap, tissue smear or scrape, or tissue biopsy can be used to obtain a sample. Thus, the sample can be a biopsy specimen (*e.g.*, tumor, polyp, mass (solid, cell)), aspirate, smear or blood sample. The sample can be a tissue that has a tumor (*e.g.*, cancerous growth) and/or tumor cells, or is suspected of having a tumor and/or tumor cells. For example, a tumor biopsy can be obtained in an open biopsy, a procedure in which an entire (excisional biopsy) or partial (incisional biopsy) mass is removed from a target area. Alternatively, a tumor sample can be obtained through a percutaneous biopsy, a procedure performed with a needle-like instrument through a small incision or puncture (with or without the aid of a imaging device) to obtain individual cells or clusters of cells (*e.g.*, a fine needle aspiration (FNA)) or a core or fragment of tissues (core biopsy).

[00141] The samples can be examined cytologically (*e.g.*, smear), histologically (*e.g.*, frozen or paraffin section) or using any other suitable method (*e.g.*, molecular diagnostic methods). A tumor sample can also be obtained by *in vitro* harvest of cultured human cells derived from an individual's tissue. Tumor samples can, if desired, be stored before analysis by suitable storage means that preserve a sample's protein and/or nucleic acid in an analyzable condition, such as quick freezing, or a controlled freezing regime. If desired, freezing can be performed in the presence of a cryoproteetant, for example, dimethyl sulfoxide (DMSO), glycerol, or propanediol-sucrose. Tumor samples can be pooled, as appropriate, before or after storage for purposes of analysis.

[00142] The antibodies of the present invention can include a label, such as, for example, a detectable label that permits detection of the antibody, and proteins bound by the antibody (*e.g.*, VISTA), in a biological sample. A detectable label is particularly suitable for diagnostic applications. For example, a VISTA antibody can be labeled with a radioactive isotope (radioisotope), which can be detected by one of skill in the art using a gamma counter, a scintillation counter or by autoradiography or other suitable means. Isotopes which are useful for the purpose of the present invention include, but are not limited to: ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe and ^{75}Se.

[00143] Antibodies of the invention can also be labeled with a fluorescent compound (*e.g.*, dyes). When the fluorescently-labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to the fluorescence of the compound. Among the most commonly used fluorescent labels are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. The antibodies of the invention can also be labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA), tetraaza-cyclododecane-tetraacetic acid (DOTA) or ethylenediaminetetraacetic acid (EDTA).

[00144] The antibodies of the present invention also can be coupled to a chemiluminescent compound. Examples of useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

[00145] Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Useful bioluminescent compounds for purposes of labeling antibodies are luciferin, luciferase and aequorin.

[00146] Detection of the labeled antibodies can be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of the enzymatic reaction of a substrate to similarly prepared standards.

[00147] In some embodiments, the approximate amount of VISTA protein in a sample is also determined. For instance, the intensity of the label from the sample can be compared to that of a known standard or control sample. Estimating the amount of a detectable target (*e.g.*, VISTA protein) in a sample is helpful, for instance, in a variety of diagnostic tests, and the estimate may be used to plan a course of treatment for a suspected disease or condition. Several commercial densitometry software programs and related instruments are available to quantitate the intensity of a stained target in a sample, such as those available from Fuji Film, Applied Biosystems, and Molecular Dynamics.

[00148] Various properties and sequence information for select anti-VISTA antibodies of the invention are provided in Tables 1A, 1B and 2 herein.

Table 1A: CDR Sequences of Select Fully Human or Humanized anti-human VISTA antibodies

mAb ID	VH family	Heavy-chain cdr1 (Imgt)	Heavy-chain cdr2 (Imgt)	Heavy-chain cdr3 (Imgt)	Light-chain cdr1 (Imgt)	Light-chain cdr2 (Imgt)	Light-chain cdr3 (Imgt)
VSTB50	B	GYTFTNYG (SEQ ID NO:1)	INPYTGEP (SEQ ID NO:2)	AREGYGNYIFPY (SEQ ID NO:3)	ESVDTYANSL (SEQ ID NO:4)	RAS (SEQ ID NO:5)	QQTNEDPRT (SEQ ID NO:6)
VSTB53		GYTFTHYT (SEQ ID NO:7)	IIPSSGYS (SEQ ID NO:8)	ARGAYDDYYDYYAMDY (SEQ ID NO:9)	QTIVHSNGNTY (SEQ ID NO:10)	KVS (SEQ ID NO:11)	FQASHVPWT (SEQ ID NO:12)
VSTB60	B	GYTFTNYG (SEQ ID NO:13)	INTYTGES (SEQ ID NO:14)	ARDYYGIYVSAY (SEQ ID NO:15)	ESVDNYANSF (SEQ ID NO:16)	RAS (SEQ ID NO:17)	QQSHEDPYT (SEQ ID NO:18)
VSTB95		GFTFRNYG (SEQ ID NO:19)	IISGGSYT (SEQ ID NO:20)	ARIYDHDGDYYAMDY (SEQ ID NO:21)	QSIVHSNGNTY (SEQ ID NO:22)	KVS (SEQ ID NO:23)	FQGSHVPWT (SEQ ID NO:24)
VSTB112	D	GGTFSSYA (SEQ ID NO:25)	IIPIFGTA (SEQ ID NO:26)	ARSSYGWSYEFDY (SEQ ID NO:27)	QSIDTR (SEQ ID NO:28)	SAS (SEQ ID NO:29)	QQSAYNPIT (SEQ ID NO:30)
VSTB116	D	GGTFSSYA (SEQ ID NO:31)	IIPIFGTA (SEQ ID NO:32)	ARSSYGWSYEFDY (SEQ ID NO:33)	QSINTN (SEQ ID NO:34)	AAS (SEQ ID NO:35)	QQARDTPIT (SEQ ID NO:36)

Table 1B: Heavy and Light Chain Sequences of Select Fully Human or Humanized anti-human VISTA antibodies

Protein ID	Heavy-chain AA CDS	Light-chain AA CDS
VSTB50	QVQLVQSGSELKKPGASVKVSCKASGYTFTNYGLNWVRQAPGQGLEWMGWINPYTGEPTYADDFKGRFVFSLDTSVSTAYLQICSLKAEDTAVYYCAREGYGNYIFPYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:47)	DIVMTQTPLSLSVTPGQPASISCRASESVDTYANSLMHWYLQKPGQPPQLLIYRASNLESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQQTNEDPRTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:48)
VSTB53	QVQLVQSGAEVKKPGASVKVSCKASGYTFTHYTIHWVRQAPGQGLEWMGYIIPSSGYSEYNQKFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGAYDDYYDYYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:49)	DIVMTQSPLSLPVTPGEPASISCRSSQTIVHSNGNTYLEWYLQKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQASHVPWTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:50)
VSTB60	QVQLVQSGSELKKPGASVKVSCKASGYTFTNYGMTWVRQAPGQGLEWMGWINTYTGESTYADDFKGRFVFSLDTSVSTAYLQICSLKAEDTAVYYCARDYYGIYVSAYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:51)	DIVMTQTPLSLSVTPGQPASISCRASESVDNYANSFMHWYLQKPGQSPQLLIYRASNLESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQQSHEDPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:52)
VSTB95	EVQLVESGGGLVQPGGSLRLSCAASGFTFRNYGMSWVRQAPGKGLEWVASIISGGSYTYYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARIYDHDGDYYAMDYWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:53)	DIVMTQSPLSLPVTPGEPASISCRSSQSIVHSNGNTYLEWYLQKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPWTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:54)

VSTB112	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:55)	DIQMTQSPSSLSASVGDRVTITCRASQSIDTRLNWYQQKPGKAPKLLIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYNPITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:56)
VSTB116	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:57)	DIQMTQSPSSLSASVGDRVTITCRASQSINTNLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQARDTPITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:58)
VSTB140*	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPAAASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:59)	DIQMTQSPSSLSASVGDRVTITCRASQSIDTRLNWYQQKPGKAPKLLIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYNPITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:56)
VSTB149*$^{\Delta}$	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPPVAGPDVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:60)	DIQMTQSPSSLSASVGDRVTITCRASQSIDTRLNWYQQKPGKAPKLLIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYNPITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:56)
VSTB174*	QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP	DIQMTQSPSSLSASVGDRVTITCRASQSIDTRLNWYQQKPGKAPKLLIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYNPITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN

	KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:61)	ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:56)

*Constant region sequences in VSTB140, VSTB149 and VSTB174 are underlined. $^{\Delta}$Amino acid residues conferring protease resistance in the heavy chain of VSTB149 are indicated in bold.

[00149] Table 2: Dissociation constant (K_D) for select anti-VISTA antibodies

Sample	KD (M)	ka1 (1/Ms)	kd1 (1/s)			
S1	1.71E-10	1.69E+06	2.89E-04	1.09E-10	1.11E+06	1.21E-04
S40	5.07E-10	1.46E+05	7.40E-05	6.96E-10	1.39E+05	9.69E-05
S41	6.32E-10	4.82E+05	3.05E-04	3.10E-10	7.08E+05	2.19E-04
S42	1.04E-10	1.05E+06	1.09E-04	2.65E-10	5.13E+05	1.36E-04
S43	2.64E-11	1.25E+06	3.30E-05	5.28E-11	1.18E+06	6.22E-05
S44	2.53E-11	1.23E+06	3.12E-05	6.40E-11	9.93E+05	6.36E-05
S45	2.35E-11	1.58E+06	3.72E-05	2.58E-11	1.46E+06	3.77E-05
S46	1.06E-10	1.56E+06	1.66E-04	2.96E-10	1.50E+06	4.44E-04
S47	3.56E-10	5.14E+05	1.83E-04	2.52E-10	5.69E+05	1.43E-04
S33	8.30E-10	1.23E+06	1.02E-03	1.22E-09	8.96E+05	1.10E-03
S34	1.08E-09	5.95E+05	6.43E-04	2.80E-09	5.20E+05	1.46E-03
S35	8.06E-11	2.08E+06	1.68E-04	1.35E-10	1.78E+06	2.41E-04
S36	6.29E-11	1.77E+06	1.12E-04	2.90E-11	1.58E+06	4.58E-05
S37	2.23E-09	5.10E+05	1.14E-03	4.43E-09	3.94E+05	1.75E-03
S38	2.26E-09	5.18E+05	1.17E-03	2.03E-09	5.37E+05	1.09E-03
S39	5.62E-10	3.97E+05	2.23E-04	3.47E-10	4.15E+05	1.44E-04
S25	1.31E-09	6.21E+05	8.12E-04	1.10E-09	5.65E+05	6.24E-04
S26	No Binding			3.53E-09	2.38E+05	8.41E-04
S27	1.13E-09	8.86E+05	9.97E-04	1.61E-09	7.12E+05	1.15E-03
S48	3.12E-10	1.24E+06	3.87E-04	1.21E-09	8.78E+05	1.06E-03
S28	2.03E-09	1.08E+06	2.19E-03	2.03E-09	9.30E+05	1.88E-03
S29	3.78E-11	1.42E+06	5.38E-05	8.90E-11	9.06E+05	8.06E-05
S30	No Binding			No Binding		
S31	Weak Binding			Weak Binding		
S32	Weak Binding			Weak Binding		
S15	9.34E-11	6.46E+05	6.04E-05	5.13E-10	3.50E+05	1.80E-04
S16	1.26E-10	5.54E+05	6.99E-05	1.92E-10	4.43E+05	8.53E-05
S17	7.68E-10	9.88E+05	7.59E-04	4.10E-10	7.09E+05	2.91E-04
S18	2.28E-09	4.90E+05	1.12E-03	1.05E-09	3.13E+05	3.29E-04
S19	1.54E-09	1.02E+06	1.58E-03	2.86E-10	7.03E+05	2.01E-04

S20	1.48E-09	6.67E+05	9.85E-04	4.57E-10	6.36E+05	2.91E-04
S21	3.18E-09	3.16E+05	1.00E-03	1.34E-09	2.70E+05	3.60E-04
S22	2.98E-09	1.09E+06	3.25E-03	1.27E-09	1.25E+06	1.59E-03
S6	6.36E-10	5.28E+05	3.36E-04	3.02E-10	5.98E+05	1.80E-04
S7	6.75E-10	1.31E+06	8.87E-04	3.27E-10	1.15E+06	3.75E-04
S8	1.15E-10	1.89E+06	2.18E-04	5.97E-11	1.25E+06	7.48E-05
S9	1.67E-10	1.87E+06	3.11E-04	9.31E-11	1.27E+06	1.18E-04
S10	8.90E-11	1.55E+06	1.38E-04	4.30E-11	1.22E+06	5.27E-05
S12	4.94E-10	1.57E+06	7.76E-04	2.39E-10	1.19E+06	2.86E-04
S13	1.02E-10	1.42E+06	1.44E-04	6.46E-11	9.55E+05	6.17E-05
S14	2.02E-10	1.26E+06	2.55E-04	7.55E-11	1.12E+06	8.43E-05
S1	2.06E-10	1.60E+06	3.29E-04	8.35E-11	1.21E+06	1.01E-04
S2	1.56E-10	9.74E+05	1.52E-04	8.66E-11	7.25E+05	6.28E-05
S3	4.33E-11	9.07E+05	3.93E-05	4.89E-11	7.41E+05	3.63E-05
S4	1.52E-10	8.98E+05	1.36E-04	7.54E-11	6.93E+05	5.23E-05
S49	1.45E-10	1.01E+06	1.46E-04	1.04E-10	7.28E+05	7.60E-05
S5	2.13E-10	1.25E+06	2.67E-04	1.37E-10	8.51E+05	1.17E-04

[00150] EXAMPLES

[00151] EXAMPLE 1: ANALYSIS OF VISTA EXPRESSION ON HUMAN HEMATOPOIETIC CELLS

[00152] Methods:

[00153] Preparation and Staining of Fresh Human PBMCs For VISTA Expression

[00154] Expression of VISTA was tested on freshly isolated human PBMCs (peripheral blood mononuclear cells) from several donors. Anti-Human VISTA-biotin (GA-1) was used for staining (5 μg/ml). Mouse IgG1, K-biotin (Clone MOPC-21 at 5 μg/ml) was used as an isotype control.

[00155] Donor Material

[00156] Blood samples were obtained from Biological Specialty Corp. (Colmar, PA) and were collected and analyzed the same day. 10 ml of whole blood containing heparin sulfate were couriered for analysis.

[00157] Sample Preparation

[00158] Blood was diluted 1:1 in sterile PBS. 22 ml diluted cord blood was layered onto 20ml sterile Ficoll-Hypaque (GE Healthcare Cat# 17-144003) in 50 ml conical tubes. Tubes were centrifuged at 1800 rpm for 20 minutes at room temperature. Mononuclear cells at the interface following centrifugation were harvested using a 1 ml pipettor and combined into two 50 ml conical tubes. Sterile PBS was added to each tube to make the volume up to 50 ml and the cells were centrifuged at 300g for 10 minutes at 4°C. Supernatant was discarded. Cells were resuspended in 50 ml of sterile PBS and tubes were spun at 300g for 10 minutes at 4°C. Supernatant was discarded. Cells were combined and resuspended in 50 ml sterile PBS prior to counting.

[00159] Staining Protocol: A frozen vial containing $5x10^7$ PBMCs was used for compensation controls and as a control for staining.

[00160] The following reagents and/or consumables were used:

[00161] FACS Stain Buffer (BSA) from BD Biosciences (Cat# 554657) supplemented with 0.2% EDTA; Phosphate-Buffered saline (PBS) (Gibco cat#14190); 96-well polypropylene round-bottomed plate (BD #3077); 1.2 ml polypropylene cluster tubes (Corning #4451); biotinylated Anti-VISTA clone GA-1 from ImmunoNext Lot# 080612B (used at 5 µg/ml); biotinylated mIgG1, K isotype control (Clone MOPC-21); Biolegend cat#400104, Lot#B116649 (used at 5 µg/ml); anti-human antibodies (See staining table below); near-Infrared live/dead dye (Invitrogen, cat# L10119); and streptavidin reagents including STP-APC (BD Biosciences cat#554067, Lot#04251) (used at 1:200 dilution in FACS buffer), STP-PE (Biolegend cat# 405203, Lot#B139688) (used at 1:200 dilution in FACS buffer), STP-PE Cy7 (showed non-specific binding in isotype control samples), STP-Q605 (Invitrogen cat# Q10101MP, Lot#53449A) (used at 1:200 dilution in FACS buffer).

[00162] Cell Surface Staining Protocol

[00163] Prior to staining, $1x10^6$ cells were transferred into 96-well round-bottomed plates and were washed with 150 µl PBS. Plates were then centrifuged at 1300 rpm at 4°C for 3 minutes.

[00164] Subsequently, cells were washed again in PBS and centrifuged as described above.

[00165] Live/dead staining was then performed in 50 µl PBS containing 0.25 µl of near-infrared live/dead dye. After 10 minutes at room temperature the wells were washed with 150 µl FACs staining buffer and centrifuged at 1300 rpm at 4°C for 3 minutes. Supernatant was discarded.

[00166] Cells were blocked with human serum at 1:100 in 50 µl FACS staining buffer. Plates were incubated at 4°C for 15 minutes. Wells were then washed with 150 µl FACs staining buffer and centrifuged at 1300rpm at 4°C for 3 minutes. Supernatant was discarded.

[00167] A cocktail containing the following antibodies was then added to each well for surface staining: The cocktails are described in Tables 3-6 below. Each cocktail would be utilized separately from the others depending on the populations of interest.

[00168] Table 3: Lineage Stain

		Target								
Fluoro	Antigen	Mouse	Rat	Human	Isotype	Clone	Supplier	Cat No.	Lot No.	Titer (μl/10^6 Cells)
FITC/AF488	CD19			X	mIgG1	HIB19	Biolegend	302206	B123019	2
PE	CD11b			X	mIgG1, K	ICRF44	BD Bio.	555388	45134	2
PerCP-Cy5.5	HLA-DR			X	mIgG2a, K	G46-6	BD Bio.	560652	25161	0.5
PE Cy7	CD16			X	mIgG1, K	3G8	BD Bio.	557744	87825	0.2
APC Cy7	NIR Live/Dead			X						
AF700	CD56			X	mIgG1, K	B159	BD Bio.	557919	19470	1
APC/AF647	VISTA-Bio			X						
PB/V450	CD3			X	mIgG1, K	UCHT1	BD Bio.	558117	90926	0.5
Q605	CD14			X	mIgG2a, K	TuK4	Invitrogen	Q10013	1049158	0.2

[00169] Table 4: T Cell Stain

		Target								
Fluoro	Antigen	Mouse	Rat	Human	Isotype	Clone	Supplier	Cat No.	Lot No.	Titer (μl/10^6 Cells)
FITC/AF488	CD4			X	mIgG1, K	RPA-T4	BD Bio.	555346	38460	2
PE	VISTA-Bio			X						
PerCP-Cy5.5	CD8			X	mIgG1, K	RPA-T8	BD Bio.	560662	1037	0.5
PE Cy7	CD56			X	mIgG1, K	B159	BD Bio.	557747	47968	0.5
APC Cy7	NIR			X						
AF700	CD45RO			X	mIgG2a, K	UCHL1	Biolegend	304218	B143062	1
APC/AF647	TCRgd			X	mIgG, K	B1	Biolegend	331212	B126473	2
PB/V450	CD45RA			X	mIgG2b, K	HI100	BD Bio.	560363	90928	0.5
Q655	CD3			X	mIgG2a	S4.1	Invitrogen	Q10012	982352	0.5

[00170] Table 5: DC Stain

		Target								
Fluoro	Antigen	Mouse	Rat	Human	Isotype	Clone	Supplier	Cat No.	Lot No.	Titer (μl/10^6 Cells)
FITC/AF488	Lin1			X	Mix	Mix	BD Bio.	340546	2152758	5
PE	CD11c			X	mIgG1, K		BD Bio.	555392	45123	2
PerCP-Cy5.5	HLA-DR			X	mIgG2a, K	G46-6	BD Bio.	560652	25161	0.5
APC Cy7	NIR			X						
APC/AF647	CD83			X	mIgG1, K	HB15e	BD Bio.	551073	57688	2
BV421	CD123			X	mIgG1, K	6H6	Biolegend	306017	B148193	0.5
Q605	VISTA-Bio			X						

[00171] Table 6: Myeloid Stain

		Target								
Fluoro	Antigen	Mouse	Rat	Human	Isotype	Clone	Supplier	Cat No.	Lot No.	Titer (μl/10^6 Cells)
FITC/AF488	CD33			X	mIgG1	HM3-4	Biolegend	303304	B100963	3
PE	CD11b			X	mIgG1, K	ICRF44	BD Bio.	555388	45134	2
APC Cy7	NIR			X						
APC/AF647	VISTA-Bio			X						
Q605	CD45			X	mIgG1, K	HI30	Invitrogen	Q10051	880470	1

[00172] Following the surface staining, cells were washed twice as previously described with FACS staining buffer and centrifuged at 1300 rpm at 4°C for 5 minutes. Samples were resuspended in 50 μl of FACS staining buffer containing the appropriate fluorescently-labeled streptavidin. Samples were incubated at 4°C for 30 minutes. Cells were washed with 150 μl FACS staining buffer and centrifuged at 1300 rpm at 4°C for 5 minutes. This wash step was repeated before samples were resuspended in 250 μl of FACS staining buffer. Samples were analyzed on a BD LSRFortessa™ cell analyzer (BD Biosciences) the same day.

[00173] Data Analysis

[00174] Flow cytometry data was reanalyzed using FlowJo Version 9 software to gate specific phenotypic populations. Enumeration of geometric mean was used to compare VISTA expression in different cell subsets. Each population was normalized for background

by subtracting isotype control values from the mean values of the anti-VISTA treated samples. Graphs were prepared in Prism and statistics were performed using either student's T-test if only two samples were compared, or one-way ANOVA with Bonferroni post-tests.

[00175] Results:

[00176] Expression of VISTA on Human Myeloid and Lymphoid Subsets:

[00177] As shown in Figures 2A-2E, 3A-3G, 4, 5A-5B and 6A-6C, VISTA expression on $CD14^+$ monocytes was significantly different from all other populations ($p < 0.001$). No significant differences between other populations were seen. Monocytes expressed the highest levels of VISTA in peripheral blood, with the $CD14^+CD16^-$ subset having significantly higher expression than $CD14^{lo}CD16^+$ cells. While APCs showed moderate expression of VISTA, lymphoid subsets showed low expression levels.

[00178] Expression of VISTA on Human T and NK Subsets:

[00179] As shown in Figures 7A-7E, 8A-8G and 9, with NK subsets, $CD56^{lo}$ cells exhibited significantly higher expression levels of VISTA than $CD56^{Hi}$ NK cells. Of T cell subsets, $CD8^+$ memory cells expressed the highest expression levels, although they are not significantly higher than $CD8^+$ naive or $CD4^+$ T cells.

[00180] Expression of VISTA on Human Dendritic Cell Subsets:

[00181] As shown in Figures 10A-10D, 11A-11C and 12, no significant differences in VISTA expression seen; DCs and basophils exhibited low expression of VISTA, with plasmacytoid dendritic cells (pDCs) generally being higher but not to a significant extent.

[00182] Conclusion: These results show expression of VISTA on various immune cell subsets, and that VISTA is expressed on monocytes most highly, with some expression on different T cell subsets and NK cells, and little to no expression on B cells.

[00183] EXAMPLE 2: VISTA EXPRESSION ON PERIPHERAL BLOOD CELLS

[00184] Methods:

[00185] Staining of whole blood: Freshly isolated whole blood (100 µl) was stained with antibody cocktails as indicated below by incubation for 30 minutes at 4°C. Red blood cells (RBCs) were lysed with RBC lysis buffer and the remaining cells were washed 1x with staining buffer. Cells were re-suspended in 200 µl of staining buffer. The data were collected using a MACSQuant flow cytometer and analyzed using FlowJo analysis software.

[00186] Staining of peripheral blood mononuclear cells (PBMCs): Peripheral blood mononuclear cells were isolated from whole blood using Ficoll gradient. Freshly isolated

1x10^6 PBMCs were stained with antibody cocktails in 100 µl of staining buffer. Samples were incubated for 30 minutes at 4°C then washed once with staining buffer. Cells were re-suspended in 100 µl of staining buffer. The data were collected using MACSQuant® flow cytometer (Miltenyi Biotec) and analyzed using FlowJo analysis software.

[00187] The antibodies used were CD11b, CD33, CD177, CD16, CD15, CD14, CD20, HLADR, CD3, CD4, CD8, CD127, CD69, and FOXP3 antibodies (Biolegend, San Diego, CA). The APC-conjugated mouse anti-human VISTA (clone GG8) was made by ImmuNext (Lebanon, NH).

[00188] Conclusions:

[00189] Expression of VISTA on healthy human peripheral blood cells

[00190] Whole blood and peripheral blood mononuclear cells were analyzed for VISTA expression using multicolor flow cytometry. As shown in Fig. 15A and 15B, the highest level of VISTA expression was detected on monocytes followed by neutrophils. Both the CD4+ and CD8+ T cells expressed low level of VISTA as shown in Figure 13C and 13D.

[00191] Expression of VISTA on cancer patient peripheral blood cells

[00192] As shown in Figures 14A-C, peripheral blood mononuclear cells (PBMCs) from lung cancer patients were analyzed. Figure 14A is a representative flow plot showing analysis of $CD14^+$ monocytes and $CD15^+$ myeloid derived suppressive cells (MDSCs). The results suggest that phenotypically $CD15^+$ cells are neutrophil derived MDSCs. Additionally, these cells are absent in healthy blood samples. Figure 14B is a representative histogram of VISTA expression on healthy and cancer patient derived monocytes, suggesting a higher level of VISTA expression on cancer patient cells compared to healthy controls. Similarly higher level of VISTA was found on MDSCs in cancer patients, as shown in Figure 14C.

[00193] Figure 15A is a representative FACS plot showing the presence of neutrophil derived MDSCs in the blood of colon cancer patients. Figure 15B and 15C are representative histograms showing higher level of VISTA expression on cancer patients' monocytes compare to healthy donor blood samples.

[00194] Expression of VISTA on cynomolgus monkey peripheral blood cells

[00195] As shown in Figure 16A and 16B flow cytometry analysis of monkey whole blood revealed the VISTA expression pattern similar to human cells. Both monocytes and neutrophils expressed the highest level of VISTA compared to $CD4^+$ (Figure 16C) and $CD8^+$ (Figure 16D) T cells.

[00196] EXAMPLE 3: VISTA EXPRESSION IN HEME MALIGNANCY CELL LINES AT THE RNA LEVEL AND PROTEIN LEVEL

[00197] Because VISTA is expressed in heme malignancies, an anti-VISTA antibody could potentially target the malignant cells for destruction, as well as block VISTA and promote anti-tumor immune responses.

[00198] The data includes RNAseq analysis of ~140 heme malignancy cell lines (some cell lines are repeated in the analysis). The data is shown in Figure 17.

[00199] The RNAseq values are listed as FPKM (Fragments Per Kilobase of exon per Million fragments mapped) values.

[00200] In essence, this means that all reads falling in the exonic regions of a gene were counted and normalized by both the length of the gene and the total number of reads per sample (to account for inter-sample differences). The cutoff value is 1; above 1 is positive for VISTA expression (at the RNA level), below 1 is negative for VISTA expression.

[00201] The results indicated that many cell lines are positive at the RNA level, primarily acute myeloid leukemias and chronic myelogenous leukemias. This may be expected since VISTA is highly expressed in normal myeloid cells, and because its function is believed to dampen immune responses, including anti-tumor immune responses.

[00202] EXAMPLE 4: GENERATION OF MONOCLONAL ANTIBODIES AGAINST VISTA

[00203] Phage Panning

[00204] Twenty four phage panning experiments were carried out to enrich for phage reactive to Cyno VISTA-His. The cynomolgus VISTA protein was used for these experiments as it showed better biotin conjugation than the human VISTA protein. To determine the success of the phage experiments, phage pools from the individual panning rounds were added to neutravidin plates coated with biotinylated cyno VISTA-His and detected with a HRP-conjugated anti-M13 antibody. Individual colonies were picked from the phage selection rounds and Fabs proteins were produced in 96 well plates. The expressed Fab supernatants were assayed for binding to biotinylated cyno VISTA-His. This resulted in more than 200 hits.

[00205] The VH and VL regions from the Fab plates were amplified, submitted for DNA sequencing and were exported as FASTA files. When picking the clones that should be converted and tested as MABs, the clones were chosen based on sequence diversity as well as having limited post-translational modification risks and as few hydrophobic residues as possible.

[00206] The VH and VL from the phage clones were sub-cloned into mammalian IgG1/kappa expression vectors and transfected into HEK293 cells. The antibodies were purified on Protein A Sepharose Fast Flow affinity resin. The concentration of the phage MABs was determined by quantitative ELISA using Nanodrop measurements. The antibody panel was expressed at high levels. SDS-PAGE analysis demonstrated the integrity of each expressed antibody variant.

[00207] In-line maturation of the phage antibodies was done by amplifying the VH domains from the polyclonal antibody mixes from the last round of panning for cloning into phage vectors that have diversity in the VL. This resulted in an enriched VH pool which was sampled with additional diversity in the VL. The phage were taken through 1-2 rounds of stringent panning with the expectation to identify very high affinity binders to VISTA ECD His protein. A monoclonal Fab ELISA was run to determine the success of the maturation. ELISA and expression data was normalized to a reference clone set to 100% from the original de novo panning experiment and affinity matured clones with higher binding signal to cyno VISTA antigen than the reference clone were identified. This process generated several clones that demonstrated up to 200% binding when screened at low antigen concentration (1 nM), the clones with highest affinity were sequenced and produced as MABs.

[00208] Hybridoma generation

[00209] One group of BALB/cAnNCrl mice received one intraperitoneal (IP) injection of 50 μg Hu VISTA-Ig recombinant protein (Sino) emulsified in Complete Freund's Adjuvant followed two weeks later by one IP injection of 50 μg Hu VISTA-Ig recombinant protein emulsified in Incomplete Freund's Adjuvant. Two weeks later the mice received one IP injection of 50 μg cyno VISTA-Fc recombinant protein emulsified in Incomplete Freund's Adjuvant. All mice received a final injection of 25 μg human and 25 μg cyno VISTA at the base of tail in PBS, five days prior to splenic harvest for fusion.

[00210] Another group of BALB/cAnNCrl mice received one IP injection of 50 μg Hu VISTA-His recombinant protein emulsified in Complete Freund's Adjuvant. Two weeks later the mice received one IP injection of 50 μg Hu VISTA-His recombinant protein emulsified in Incomplete Freund's Adjuvant. Two weeks later the mice received one IP injection of 50 μg

Cyno VISTA-His recombinant protein emulsified in Incomplete Freund's Adjuvant. Two weeks later all mice received a final injection of 25 µg Hu VISTA-His and 25 µg Cyno VISTA-His in PBS, three days prior to splenic harvest for fusion.

[00211] On the day of fusion, mice were euthanized by CO2 asphyxiation; the spleens were removed and placed into 10 mL of cold phosphate-buffered saline. A single cell suspension of splenocytes was prepared by grinding spleens through a fine mesh screen with a small pestle and rinsing with PBS at room temperature. Cells were washed once in PBS and subjected to RBC lysis. Briefly, cells were resuspended in 3mL of RBC lysis buffer (Sigma #R7757) per every spleen and placed on ice for 5 minutes. Cells were again washed once in PBS at room temperature and labeled for magnetic sorting. As per manufacturer's instructions, cells were labeled with anti-murine Thy1.2, anti-murine CD11b and anti-murine IgM magnetic beads (Miltenyi Biotec # 130-049-101, 130-049-601 and 130-047-301 respectively) then sorted using a MS column with a Midi MACS. The negative cell fractions (positive cell fractions were discarded) were fused to FO cells. Fusion was carried out at a 1:1 ratio of murine myeloma cells to viable spleen cells. Briefly, spleen and myeloma cells were mixed together, pelleted and washed once in 50 mL of PBS. The pellet was resuspended with 1 mL of polyethylene glycol (PEG) solution (2 g PEG molecular weight 4000, 2 mL DMEM, 0.4 mL DMSO) per 10e8 splenocytes at 37°C for 30 seconds. The cell/fusion mixture was then immersed in a 37°C water bath for approximately 60 seconds with gentle agitation. The fusion reaction was stopped by slowly adding 37°C DMEM over 1 minute. The fused cells were allowed to rest for 5 minutes at room temperature and then centrifuged at 150 x g for 5 minutes. Cells were then resuspended in Medium E-HAT (MediumE (StemCell Technologies cat#03805) containing HAT (Sigma cat#H0262) and seeded in 96-well flat bottom polystyrene tissue culture plates (Corning # 3997).

[00212] A capture EIA was used to screen hybridoma supernatants for antibodies specific for cyno VISTA. Briefly, plates (Nunc-Maxisorp #446612) were coated at 4µg/ml for at least 60 minutes with goat anti-mouse IgG (Fc) antibody (Jackson #115-006-071) in coating buffer (Thermo 28382). Plates were blocked with 200 µl/well of 0.4% (w/v) bovine serum albumin (BSA) in PBS at for 30 minutes at RT. Plates were washed once and 50 µl/well of hybridoma supernatant was added and incubated at room temperature for at least 30 minutes. Plates were washed once and 50 µl/well of 0.1 µg/mL of cyno VISTA-huIg was added and incubated at RT for 30 minutes. Plates were washed once and 1:40,000 Streptavidin HRP (Jackson 016-

030-084) in 0.4% BSA/PBS was added to plates and incubated for 30 minutes at RT. Plates were washed 3x and subsequently developed using 100µl/well TMB Turbo substrate (Thermo Scientific 34022) incubating approximately 10 minutes at RT. The reaction was stopped using 25µl/well 4N Sulfuric Acid and absorbance was measured at 450 nm using an automated plate spectrophotometer. Fifteen of the primary hits were selected for subcloning by limiting dilution and were screened in the same primary screen format.

[00213] All cyno VISTA reactive hybridoma cell lines were cross screened using human VISTA-Ig to assess cross-reactivity. Briefly, plates (Nunc-Maxisorp #446612) were coated at 4µg/mL with goat anti-ms Fc (Jackson#115-006-071) in 0.1M sodium carbonate-bicarbonate buffer, pH 9.4 (Pierce 28382 BupH™) O/N at 4°C. Without washing, the wells were blocked with 200 µl of block (0.4% BSA (Sigma) (w/v) in PBS (Invitrogen)) overnight at 4°C. After removing block solution, undiluted hybridoma supernatants were incubated on coated plates for 30 minutes at RT. Plates were washed once with PBST (0.02% Tween 20 (Sigma) (w/v) in PBS), and then incubated for 30 minutes with Hu VISTA-Ig diluted to 100 ng/ml in block. Plates were washed once with and probed with Goat antihuman-Fc-HRP (Jackson #109-036-098) diluted 1:10,000 in block for 30 minutes at RT. Plates were again washed and subsequently developed using 100µl/well TMB Turbo substrate (Thermo Scientific 34022) incubating approximately 10 minutes at RT. The reaction was stopped using 25µl/well 4N Sulfuric Acid and absorbance was measured at 450 nm using an automated plate spectrophotometer.

[00214] Hybridomas that were shown to be reactive to both human and cynomolgus VISTA had their V region antibody sequences cloned. Hybridoma cells were prepared prior to the reverse transcriptase (RT) reactions with Invitrogen's SuperScript III cells Direct cDNA System. Briefly, the culture medium was discarded and the plate placed on ice and resuspended in 200 µl cold PBS. Forty microliters was transferred to a MicroAmp fast 96 well Reaction PCR plate and the plate was placed on a cold metal plate base, sealed with plastic film and spun at 700 rpm for 3 minutes. The PBS was discarded and to each well, 10 µl Resuspension Buffer and 1 µl Lysis Enhancer was added. The plate was sealed and incubated at 75°C for 10 min and stored at -80°C.

[00215] For the RT reaction, each well contained 5 µl water, 1.6 µl 10X DNase Buffer, 1.2 µl 50 mM EDTA, 2 µl Oligo(dT)20 (50 mM) and 1 µl 10 mM dNTP Mix. The plate was incubated at 70°C for 5 min, followed by incubation on ice for 2 min, then the following reagents were added for each well; 6 µl 5X RT Buffer, 1µl RNaseOUT™ (40 U/µl), 1 µl SuperScript™ III RT (200 U/µl) and 1 µl of 0.1M DTT. The plate was sealed and placed on a thermal cycler preheated to 50°C and incubated at 50°C for 50 minutes, followed by inactivation (5 min incubation at 85°C). The reaction was chilled on ice and the single-stranded cDNA was stored at –80°C until further use.

[00216] For V region amplifications, 20 µl PCR reactions were set up. Each well contained 16.2 µl water, 2.0 µl 10X PCR Reaction buffer, 0.8 µl MgSO4 (50 mM), 0.4 µl 10mM dNTP, 0.15 µl 100 uM Forward primer mix 0.05 µl 100 uM Reverse primer, 0.2 µl HiFi Tag enzyme. The cDNA, prepared as described above, was transferred (2 µl/well) to the PCR components mixture, the plate was sealed and an amplification reaction was run; for VH the program was (i) 94°C for 1 min (ii) 94°C for 15 sec (iii) 55°C for 30 sec (iv) 68°C for 1 min. Steps (ii – iv) were repeated for a total of 35 cycles followed by a final extension at 68°C for 3 min. for VL the program was (i) 94°C for 1 min (ii) 94°C for 15 sec (iii) 55°C for 30 sec (iv) 65°C for 30 sec, (v) 68°C for 1 min. Steps (ii – v) were repeated for a total of 35 cycles followed by a final extension at 68°C for 3 min.

[00217] Forward primers were pre-mixed and such mixture was used in ration 3:1 with the reverse primer. PCR products were verified on an agarose gel. The reactions were prepared for infusion cloning by the addition of Enhancer (In-Fusion HC Cloning Kit, cat #639650, Clontech). Five microliters of the PCR reaction was transferred to a PCR plate followed by the transfer of 2 µl of enhancer/well. The plate was sealed and incubated in a thermal cycler (15 min at 37°C and 15 min at 80°C). The destination vector (vDR243 or vDR301) was prepared by Esp3I digestion; (1.5µg vector was digested in 3 µl Tango Buffer, 2 1 Esp3I and water in a 30 µl reaction at 37°C for 2 hours).

[00218] For infusion cloning, 2 µl of enhancer treated PCR product was mixed with 100 ng Esp3I digested vector and 2 µl of 5X infusion enzyme (Clontech). The infusion reaction was done in 96-well PCR plate format. The plate was incubated for 15 min at 50°C on a PCR machine and Stella competent cells were transformed by heat shock for 40 seconds at 42°C without shaking and spread on LB agar plates with select antibiotic and incubated overnight at 37°C. Next day, colonies were picked into 96-well deep well plates containing LB/Carbenicillin media and grown overnight at 37°C. Frozen stocks were made from overnight culture mixing with equal volume of 30% w/v glycerol. The V regions were sequenced using sequencing primer SPF0052. The sequences were analyzed, one positive well per hybridoma vH and vL was chosen, re-arrayed in new plates and grown overnight in rich medium with ampicillin. Clones then had miniprep DNA prepared for small scale transfection in 96-well plate.

[00219] Forty eight selected mouse hybridoma sequences for both heavy and light chain were human framework adapted using an internal software program. One human framework was chosen for each one of the mouse vH or vL. V region DNA sequences were obtained through back-translation. Synthetic DNA regions corresponding to the HFA amino acid sequences were ordered from Integrated DNA Technologies (Coralville, IA). Cloning was performed into pre-cut vDR149 and vDR157, human IgG1 and human kappa respectively. Qiagen Endo-free Maxi-prep kits were used to prepare the DNA. Expi293 (100 ml) cultures were used to express this antibody panel.

[00220] EXAMPLE 5: PROTOCOL FOR HUMAN VISTA-IG T CELL SUPPRESSION ASSAY *IN VITRO*

[00221] Mouse A20 cells were stably transfected with either GFP or human VISTA. They were incubated with ova peptide and with DO11.10 T cells. CD25 expression by the T cells was measured 24 hours after incubation began. The A20-huVISTA cells suppress CD25 expression by the T cells, but this readout is significantly restored by incubation with VSTB95 (Figure 18).

[00222] EXAMPLE 6: HUMAN FRAMEWORK REGIONS ADAPTATION OF ANTI-VISTA ANTIBODIES

[00223] Mouse hybridoma sequences for both heavy and light chain were human framework adapted by CDR-grafting (Jones, et al. *Nature*, 321: 522-525 (1986) using an internal software program. The program delineates the complementarity determining regions (CDRs) of the V region sequences according to the Kabat definitions (Wu, T. T. & Kabat, E. A. (1970). *J Exp Med,* 132, 211-50) and compares the framework regions with the human germline genes using Blast. The human germline with the highest sequence identity to the mouse frameworks was chosen as the acceptor gene for human framework adaptation (HFA). In a few cases, closely related human germline genes were chosen instead, based on previous experience with well-expressed human frameworks. DNA sequences for the human frameworks chosen for each one of the mouse vH or vL V regions were obtained through back-translation. Synthetic DNA regions corresponding to the HFA amino acid sequences were ordered from Integrated DNA Technologies (Coralville, IA). Cloning was performed into human IgG1 and human kappa, respectively.

[00224] EXAMPLE 7: ANTI-VISTA ANTIBODY CONSTRUCTS

[00225] Plasmid and sequence information for the molecules for cell line development: Plasmid constructs were generated for anti-VISTA antibodies having the VSTB112 variable regions and an IgG1κ constant regions (VSTB174, new number due to an allotypic change in the constant region), an IgG2sigma constant region (VSTB140) or an IgG1 protease-resistant constant region (VSTB149).

[00226] Lonza Vectors

[00227] The pEE6.4 and pEE12.4 Chinese hamster ovary (CHO) expression vector system (Lonza Biologics, PLC) was established in Biologics Research (BR) and Pharmaceutical Development & Manufacturing Sciences (PDMS) as the primary expression system for generation of therapeutic mAbs in mammalian expression cell lines. Each vector contains a human cytomegalovirus (huCMV-MIE) promoter to drive the expression of the heavy chain (HC) or light chain (LC) and contains the ampicillin resistence gene. pEE12.4 vector also includes the gene encoding the glutamine synthetase (GS) enzyme. Growth conditions which require glutamine synthetase activity places selective pressure on the cells to maintain the expression vector (GS Gene Expression System Manual Version 4.0). pEE6.4 was used to clone the HC gene and pEE12.4 to clone the LC gene as single gene vectors. The Lonza double gene plasmid is created from these two Lonza single genes vectors.

[00228] **Amino Acid Sequences of Variable Heavy Chain Regions of Select VISTA mAbs**

[00229] > VSTB112 heavy chain (SEQ ID NO:37)

[00230] QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSS

[00231] > VSTB50 heavy chain (SEQ ID NO:38)

[00232] QVQLVQSGSELKKPGASVKVSCKASGYTFTNYGLNWVRQAPGQGLEWMGWINPYTGEPTYADDFKGRFVFSLDTSVSTAYLQICSLKAEDTAVYYCAREGYGNYIFPYWGQGTLVTVSS

[00233] > VSTB53 heavy chain (SEQ ID NO:39)

[00234] QVQLVQSGAEVKKPGASVKVSCKASGYTFTHYTIHWVRQAPGQGLEWMGYIIPSSGYSEYNQKFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGAYDDYYDYYAMDYWGQGTLVTVSS

[00235] > VSTB95 heavy chain (SEQ ID NO:40)

[00236] EVQLVESGGGLVQPGGSLRLSCAASGFTFRNYGMSWVRQAPGKGLEWVASIISGGSYTYYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARIYDHDGDYYAMDYWGQGTTVTVSS

[00237] > VSTB116 heavy chain (SEQ ID NO:64)

[00238] QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSS

[00239] **Amino Acid Sequences of Variable Light Chain Regions of Select VISTA mAbs**

[00240] >VSTB50 light chain (SEQ ID NO:41)

[00241] DIVMTQTPLSLSVTPGQPASISCRASESVDTYANSLMHWYLQKPGQPPQLLIYRASNLESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQQTNEDPRTFGQGTKLEIK

[00242] >VSTB53 light chain (SEQ ID NO:42)

[00243] DIVMTQSPLSLPVTPGEPASISCRSSQTIVHSNGNTYLEWYLQKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQASHVPWTFGQGTKLEIK

[00244] >VSTB95 light chain (SEQ ID NO:43)

[00245] DIVMTQSPLSLPVTPGEPASISCRSSQSIVHSNGNTYLEWYLQKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPWTFGQGTKLEIK

[00246] >VSTB112 light chain (SEQ ID NO:44)

[00247] DIQMTQSPSSLSASVGDRVTITCRASQSIDTRLNWYQQKPGKAPKLLIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYNPITFGQGTKVEIK

[00248] >VSTB116 light chain (SEQ ID NO:45)

[00249] DIQMTQSPSSLSASVGDRVTITCRASQSINTNLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQARDTPITFGQGTKVEIK

[00250] EXAMPLE 8: ELISA and FACS SCREENING OF ANTI-VISTA ANTIBODIES

[00251] These experiments were to determine the ability of the produced antibodies to bind human or cynomolgus VISTA protein in an ELISA, as well as to determine, using FACS screening, the ability of the antibodies to bind VISTA protein on the surface of K562 cells (human myelogenous leukemia cell line) expressing human or cynomolgus VISTA proteins.

[00252] Methods:

[00253] ELISA procedure summary: Plates were coated overnight at 4°C with 1 μg/ml SB0361 (human) or SB0361 (cyno (cynomolgus)) proteins, which are the extracellular domains of VISTA from the respective species. Antibodies were diluted to 1 μg/ml as a starting concentration with 1:4 step-wise dilutions for a total of 4 concentrations and incubated at room temperature room temperature (RT) for 2 hours. Mouse anti-human IgG1-HRP (horseradish peroxidase) was used for detection and incubated for 1 hour at RT. All washes were performed using PBS-Tween (0.05%).

[00254] FACS procedure summary: 2 x 10^5 K562-G8 (human) or K562-C7 (cyno) cells were stained with 5 μg/ml of each test antibody and incubated for 30 minutes at 4°C. Goat anti-human IgG1-PE (phycoerythrin) antibody was used as a secondary detection antibody at 5 μg/ml. Cells were run on a BD Fortessa and analyzed using FlowJo software (Tree Star, Inc., Ashlang, OR) for MFI (mean fluorescence intensity) of the live population.

[00255] Data Analysis/Results: For each antibody, a subjective score (Yes/No) was given relating to whether the antibody bound robustly or not for both the ELISA and FACS analysis for each of the 4 assays. If an antibody gave a "No" result for binding in either assay, it was repeated to confirm that it was negative. The results are shown in Table 7 below and in Figures 19A-19F and 20A-20F.

[00256] Table 7.

INX Code	Hu ELISA	Cyno ELISA	Hu FACS	Cyno FACS
1	Y	Y	Y	Y
2	Y	Y	Y	Y
3	Y	Y	Y	Y
4	Y	Y	Y	Y
5	Y	Y	Y	Y
6	Y	Y	Y	Y
7	Y	Y	Y	Y
8	Y	Y	Y	Y

9	Y	Y	Y	Y
10	Y	Y	Y	Y
11	N	N	N	N
12	Y	Y	Y	Y
14	Y	Y	Y	Y
16	Y	Y	Y	Y
17	Y	Y	Y	Y
18	Y	Y	Y	Y
19	Y	Y	Y	Y
20	Y	Y	Y	Y
21	Y	Y	Y	Y
22	Y	Y	Y	Y
23	N	N	N	N
24	N	N	N	N
25	Y	Y	Y	Y
26	N	Y	N	Y
28	Y	Y	Y	Y
30	N	N	N	N
31	N	N	N	N
32	N	N	N	N
33	Y	Y	Y	Y
34	Y	Y	Y	Y
35	Y	Y	Y	Y
36	Y	Y	Y	Y
37	Y	Y	Y	Y
38	Y	Y	Y	Y
39	Y	Y	N	N
40	Y	Y	Y	Y
41	Y	Y	Y	Y
42	Y	Y	Y	Y
43	Y	Y	Y	Y
44	Y	Y	Y	Y
45	Y	Y	Y	Y
46	Y	Y	Y	Y
47	Y	Y	Y	Y
48	Y	Y	Y	Y
49	Y	Y	Y	Y

[00257] EXAMPLE 9: SCREENING RESULTS OF ANTI-HUMAN VISTA ANTIBODIES USING THE MIXED LYMPHOCYTE REACTION (MLR) AND *STAPHYLOCOCCUS* ENTEROTOXIN B (SEB) ACTIVATION ASSAYS

[00258] The purpose of this study was to present data supporting the identification of multiple functional α-VISTA antibodies that enhance cellular immune responses in the mixed lymphocyte reaction (MLR) assay, as well as the *staphylococcus* enterotoxin B activation (SEB) assay.

[00259] The mixed lymphocyte reaction (MLR) is a standard immunological assay that depends upon MHC class I and II mismatching to drive an allogeneic T cell response. Peripheral blood mononuclear cells are isolated from two mismatched individuals, incubated together and as a result of these mismatches, proliferation and cytokine production occurs.

[00260] Material and Methods:

[00261] 10% AB Media was prepared by combining 500 ml of RPMI with 50 ml of human AB serum, 5 ml of Penicillin/Streptomycin (10,000 U/ml), 5 ml of L-glutamine (100x) and 10 ml of HEPES (1M). Media was stored for no longer than 14 days. 1 mCi tritiated thymidine was prepared by diluting 0.2 ml of thymidine stock (1 mCi/ml) in 9.8 ml of RPMI.

[00262] Soluble VISTA antibodies were diluted to 20 μg/ml in 10% AB serum media. 100 μl of the appropriate antibody solutions was added to the appropriate wells of a 96 well U-bottom plate (Falcon product #353077 or equivalent). After the various cellular populations were added, the final concentration was 10 μg/ml.

[00263] Isolation of white blood cells: Donors were at least 18 years of age, generally healthy and selected randomly from the local population. Transferred donor blood from isolation tubes to 50 ml conicals. Under-laid 15 ml of Ficoll 1077 per 25 ml of blood being careful not to mix with the blood. Centrifuged the cells at 1250g for 25 minutes at room temperate with no brake. White blood cells were isolated at the interphase of the Ficoll and the serum and diluted the cells into 40 ml of Hanks Balances Salt Solution (HBSS). Centrifuged the cells at 453g (1500 rpm) for 10 minutes at 4°C. Resuspended the cells in 50 ml of HBSS and counted by transferring 500 μl to a separate tube.

[00264] Mixed lymphocyte reaction (MLR) 96 well plate setup: Determined the appropriate number of "stimulator cells" and "responder cells" needed for the assay based on the number of samples to be analyzed. The stimulator population is seeded at 0.5 x 10^5 cells/well and the responder population is seeded at 1.0 x 10^5 cells/well of a 96 well U-bottom plate. All conditions must be performed in triplicate. The appropriate number of "stimulator cells" were pipetted into a new conical and centrifuged as previously described. Resuspended cells in 10 ml and irradiated with 4000 rads. Centrifuged cells as previously described and resuspended at a concentration of 1 x 10^6/ml in 10% AB serum media and added 50 μl to appropriate wells. Isolated the required number of responder cells and centrifuged as previously described and resuspended at a concentration of 2 x 10^6/ml in 10% AB serum media and added 50 µl to appropriate wells. Incubated the cells for 5 days at 37°C and 5% CO_2. On the fifth day, removed 30 µl of supernatant for analysis of interferon gamma (IFN-γ) production. On the fifth day, added 25 µl of a 40 µCi/ml tritiated thymidine solution to each well and incubated for 8 hours at 37°C and 5% CO_2. Transferred cells to the 96 well micro scintillation plate per manufacturer's instructions. Counted using the micro scintillation counter per manufacturer's instructions. IFN-γ concentration was determined by ELISA (eBioscience cat# 88-7316-88) using manufacturer's protocol.

[00265] Data analysis: Calculated the average counts per minute (CPM) or IFN-γ concentration for the non-treated wells. Calculated the average CPM or IFN- γ for each of the test groups. Log_{10} transform the data set. Using 12 MLR fold-scores for each compound, calculated the average for the set of 12 test groups of each compound Average score for 12 experiments = Σ [($\log_{10}$ (Average CPM of triplicate for test compound)) - ($\log_{10}$ (Average CPM of triplicate for No Treatment))]/12

[00266] Acceptance criteria: All test reagents and appropriate controls were tested for endotoxin prior to running the assay and have levels of < 0.1 EU/mg. The responder cells alone had CPM counts below 700 CPM on average indicating that the cells were quiescent when incubated alone. The CPM for the MLR group was at least 2 fold higher than the CPM for responder cells incubated alone indicating that a reaction had occurred and that the donors are a mismatch. All MLR assays included a human IgG1 negative control protein. The result of the human IgG1 negative control was not statistically different from the non-treated samples based upon use of a student's t-test.

[00267] Screening of anti-VISTA antibodies in the MLR: Initial screen of all compounds. Prior to running the MLR with the anti-VISTA antibodies, antibodies were confirmed to bind both cell bound VISTA via FACS analysis and VISTA protein via ELISA. Antibodies S26 (VSTB77), S30 (VSTB86), S31 (VSTB88), S32 (VSTB90) and S39 (VSTB74) failed this initial screen but were still tested in the assay. For the purpose of initial screening, all antibodies were tested at 10µg/ml in the MLR with proliferation and IFN-γ being the parameters measured (Figures 21A-21D and 22A-22B).

[00268] Selection of six lead antibodies. From the initial screen, six candidates were chosen for further analysis: VSTB112 (S2), VSTB116 (S5), VSTB95 (S16), VSTB50 (S41), VSTB53 (S43) and VSTB60 (S47).

[00269] Dilution studies of the top six candidates in the MLR: Protocol adjustments. The protocol is identical as previously described with the adjustment that antibodies were diluted to the following concentrations: 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 μg/ml.

[00270] Determination of IC_{50} values: Raw CPM counts and IFN-γ concentrations were used to determine the IC_{50} for each of the antibodies. Calculations of IC_{50} were determined through use of the program "EZ-R stats." Six individual responders were used to determine the IC_{50} values. Individual CPM counts and IFN-γ concentrations in the MLR with dose titrations of the lead candidates.

[00271] Table 8: IC_{50} values for both CPM and IFN-γ in the MLR

	VSTB112 (S2)	VSTB116 (S5)	VSTB95 (S16)	VSTB50 (S41)	VSTB53 (S43)	VSTB60 (S47)
CPM	-0.67	-0.78	-0.54	-0.12	-0.33	0.02
Gamma	-0.42	-0.16	0.22	0.06	0.27	0.4

** Values are in $\log_{10}$ of antibody concentrations.

[00272] Conclusion: The initial screen indicated that multiple VISTA specific antibodies were capable of enhancing the MLR cellular immune response. Antibodies were then ranked based upon efficacy and variance and based upon these results, VSTB112, VSTB116, VSTB95, VSTB50, VSTB53 and VSTB60 were chosen to evaluate in dose-titration experiments. VSTB60 induced a weaker response than the other five antibodies in the dose-titration experiments.

[00273] The *staphylococcus* enterotoxin B (SEB) activation assay: SEB is a bacterial super-antigen that induces activation of specific Vβ+ T cells. Peripheral blood mononuclear cells are isolated and incubated with the SEB antigen in culture, which induces robust cytokine production. This assay was conducted on the five lead candidates.

[00274] Preparation of 10% AB Media, preparation of 1 mCi tritiated thymidine, preparation of soluble VISTA antibodies, and isolation of white blood cells were all performed as previous described above in the MLR.

[00275] SEB 96 well plate setup: Determined the appropriate number of responder cells needed for the assay based on the number of samples to be analyzed. The responder population is seeded at 2.0×10^5 cells/well of a 96 well U-bottom plate. All conditions must were performed in triplicate. Centrifuged cells as previously described and resuspended at a concentration of 4×10^6/ml in 10% AB serum media and added 50 μl to the appropriate wells. Added 50 μl of 10% AB serum media containing the SEB antigen at a concentration of

40ng/ml. In the described experiments, SEB was obtained from Sigma Aldrich (cat# S0812). The final concentration in the well was at 10ng/ml. Incubated the cells for 3 days at 37°C and 5% CO_2. On the third day, removed 30 μl of supernatant for analysis of IFN-γ production. Added 25 μl of a 1 mCi/ml tritiated thymidine solution to each well and incubated for 8 hours at 37°C and 5% CO_2. Cells were transferred to the 96 well micro scintillation plate per manufacturer's instructions. Counted using the micro scintillation counter per manufacturer's instructions. IFN-γ concentration was determined by ELISA (eBioscience cat # 88-7316-88) using manufacturer's protocol.

[00276] Protocol: Data analysis. Calculated the average counts per minute (CPM) or IFN-γ concentration for each of antibodies at all concentrations. Acceptance criteria were performed as previously described. Determination of IC_{50} values was performed as described. Individual CPM counts and IFN-γ concentrations in the SEB assay with dose titrations of the lead candidates.

[00277] Table 9: IC_{50} values for both CPM and IFN-γ in the SEB.

	VSTB112 (S2)	VSTB116 (S5)	VSTB95 (S16)	VSTB50 (S41)	VSTB53 (S43)	VSTB60 (S47)
CPM	-1.16	-1.44	-1.12	-0.74	-1.06	not done
Gamma	-1.24	-0.35	0.05	1.69	-1.05	not done

**Values are in $\log_{10}$ of antibody concentrations.

[00278] Conclusions: VISTA specific antibodies enhanced cytokine production and proliferation in a dose dependent manner in the SEB assay. IC_{50} values from the SEB study were generally similar to the results from the MLR dilution studies.

[00279] EXAMPLE 10: EPITOPE BINNING ASSAY

[00280] Methods: ProteOn XPR36 system (BioRad) was used to perform epitope binning. ProteOn GLC chips (BioRad, Cat#176-5011) were coated with two sets of 6 monoclonal antibodies (mAbs) using the manufacturer instructions for amine-coupling chemistry (BioRad, cat #176-2410).

[00281] Competing mAbs were pre-incubated in excess (250 nM final concentration) with human VISTA (25 nM final concentration) for 4 hours at room temperature and 6 at a time were run over the chip coated with the panels of coated mAbs with an association time of 4 minutes followed by dissociation for 5 minutes. Following each run, the chips were regenerated with 100 mM phosphoric acid.

[00282] The data analysis involved grouping all sensorgrams by ligand and applying an alignment wizard, which automatically performs an X and Y axis alignment, and artifact removal. An Interspot correction was then applied to the data.

[00283] A non-competing mAb was defined as having a binding signal the same or > A1 signal (binding to human VISTA only).

[00284] A competing mAb was defined as having binding signal << A1 signal (*i.e.*, binding to human VISTA only).

[00285] Results: In the example sensorgram shown in Figure 23, theVSTB85 antibody was coated on the Proteon SPR chip and VISTA protein preincubated with the indicated competitors was run over the chip. VSTB50 is an example of a non-competitive antibody, as a positive response was seen when the VISTA/VSTB50 complex was run. GG8, VSTB49 and VSTB51 complexed with VISTA did not bind to the VSTB85 coated on the chip and were therefore classified as competing for the same binding site on VISTA as VSTB85.

[00286] Table 10:

		Sample Set #1: coupled to sensor						Sample Set #2: coupled to sensor					
		L1	L2	L3	L4	L5	L6	L1	L2	L3	L4	L5	L6
Samples	**Group**	**GG8**	**B85**	**B95**	**B104**	**B112**	**B113**	**B50**	**B53**	**B66**	**B67**	**IE8**	**B116**
GG8	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB100.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB101.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB102.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB103.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB104.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB105.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB106.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB107.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB108.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB109.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB110.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB111.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB112.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB113.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB114.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB115.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB116.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB49.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB51.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB53.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB59.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB65.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y

VSTB67.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB70.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB81.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB92.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB95.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB97.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB98.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB99.001	1	Y	Y	Y	Y	Y	Y	N	Y	N	Y	Y	Y
VSTB50.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB54.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB56.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB60.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB63.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB66.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB73.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB76.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB78.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB84.001	2	N	N	N	N	N	N	Y	N	Y	N	N	N
VSTB85.001	3	Y	Y	Y	Y	Y	Y	N	Y	N	Y	I	Y
VSTB74.001	4	N	N	N	N	N	N	N	N	N	N	N	N
IE8	5	Y	I	Y	Y	Y	Y	N	Y	N	Y	Y	Y

mAb immobilized on sensor

Y = Yes competed (signal << than A1- human VISTA only)

N = No competed (signal > than A1- human VISTA only)

I = Inconclusive (signal similar to A1-human VISTA only)

[00287] EXAMPLE 11: PROTEON AFFINITY DETERMINATION

[00288] Antibodies were captured on ProteOn chips using anti-IgG Fc coated surfaces. The antibodies were tested for binding of human and cynomolgus (cyno) VISTA extracellular domains (ECDs) at concentrations of VISTA proteins ranging from 0.39 nM to 100 nM. The antigens were allowed to bind/associate to the antibody-coated chips for 4 minutes after which time dissociation was monitored for 30 minutes. Chips were regenerated with two treatments of 100 mM phosphoric acid for 18 seconds. All experiments were run at 25°C and data was fit to 1:1 Langmuir binding model.

[00289] EXAMPLE 12: EFFECTS OF ANTI-VISTA TREATMENT IN A MB49 MURINE BLADDER TUMOR MODEL

[00290] Methods:

[00291] C57Bl/6 mice were injected with MB49 tumor cells. Once the tumors were established, anti-VISTA treatment was initiated. Tumor growth was then monitored 3

times/week. Mice were euthanized, in accordance with IACUC regulations, once the tumors reached 15 mm in any dimension.

[00292] For each experiment, a frozen vial of MB49 cells was thawed and grown in RPMI 1640 (+ L-Glut) with 10% serum and penicillin/streptomycin antibiotics. After three days in culture, the cells were harvested using StemPro Accutase and resuspended in RPMI at a concentration of $5x10^6$ cells/ml and 50 µl injected per mouse.

[00293] Female C57Bl/6 mice, aged 6-8 weeks were purchased from the National Cancer Institute. Upon arrival they were allowed to acclimatize for one day prior to having their right flanks shaved and their tails tattooed. They were then injected three-five days later.

[00294] Tumor Injection (Intradermal): Mice were injected intradermally (i.d.) on their shaved flank with 50 µl of MB49 cell suspension (~250,000 cells).

[00295] Monitoring Tumor Growth: Tumor growth was measured using electronic calipers first across the widest dimension (L) and secondly at a 90° angle to the first measurement (W). Tumor volume derived as follows:

[00296] Volume = $(L^2 * W^2)/2$

[00297] Tumors were considered established once they reached ~5mm in diameter (~60 mm^3 volume). Once established, treatment was initiated. Tumor growth was measured three times per week over the course of treatment and until the experiment was terminated.

[00298] Anti-VISTA Treatment: Chimerized 13F3-mIgG2a monoclonal antibody was injected intraperitoneally at 10 mg/kg. Injection schedules were thrice weekly for four weeks.

[00299] Euthanizing Mice: As per IACUC requirements, animals were euthanized once their tumors reached 15mm in the longest dimension.

[00300] Analyzing Efficacy: Mouse tumor volumes were analyzed using Excel for data management, and GraphPad Prism for graphing. Statistical analysis was performed using a macro for R statistical computing software.

[00301] The experimental design is shown in Figure 24.

[00302] Results:

[00303] Ch13F3-mIgG2a treatment in female mice led to complete tumor rejection (CR) in 70% of the animals and partial remission (PR) in 30% (n=7) (Table 13 and Figure 25B). In contrast, all of the control mIgG2a-treated mice showed progressive growth of the tumors

(6/6) (Figure 25A). These data demonstrate that anti-VISTA treatment can have a profound effect on tumor growth.

[00304] Table 11: Complete remission (CR) versus partial remission (PR)

	Female 13F3 IgG2a (n=7)
CR	5
PR	2 till day 32

[00305] The human VISTA sequence is shown in Figures 26 and 27, adapted from Wang et al., 2011, *supra.*

[00306] EXAMPLE 13: EPITOPE MAPPING OF ANTI-VISTA ANTIBODIES USING HYDROGEN/DEUTERIUM (H/D) EXCHANGE STUDIES

[00307] To identify the epitopes for VSTB50, 60, 95 and 112 on human VISTA, solution hydrogen/deuterium exchange-mass spectrometry (HDX-MS) was performed using the corresponding Fabs. For H/D exchange, the procedures used to analyze the Fab perturbation were similar to that described previously (Hamuro *et al.,* J. Biomol. Techniques 14:171–182, 2003; Horn *et al.,* Biochemistry 45:8488-8498, 2006) with some modifications. Fabs were prepared from the IgGs with papain digestion and Protein A capture using Pierce Fab Preparation Kit (Thermo Scientific, Cat# 44985) . The human VISTA protein sequence contains six N-linked glycosylation sites. To improve the sequence coverage, the protein was deglycosylated with PNGase F. The deglycosylated VISTA protein was incubated in a deuterated water solution for predetermined times resulting in deuterium incorporation at exchangeable hydrogen atoms. The deuterated VISTA protein was in complex with either Fab of VSTB50, VSTB60, VSTB95 or VSTB112 in 46 µL deuterium oxide (D_2O) at 4 °C for 30 sec, 2 min, 10 min and 60 min. The exchange reaction was quenched by low pH and the proteins were digested with pepsin. The deuterium levels at the identified peptides were monitored from the mass shift on LC-MS. As a reference control, VISTA protein was processed similarly except that it was not in complex with the Fab molecules. Regions bound to the Fab were inferred to be those sites relatively protected from exchange and, thus, containing a higher fraction of deuterium than the reference VISTA protein. About 94% of the protein could be mapped to specific peptides.

[00308] The solution HDX-MS perturbation maps of VISTA with VSTB50 / VSTB60, and VSTB95 / VSTB112 are shown in Figure 28 top and bottom, respectively. Two epitope groups were identified. Anti-VISTA VSTB50 recognizes the same epitope as VSTB60 does;

VSTB95 binds to another epitope region as VSTB112 does on VISTA. Anti-VISTA VSTB50 and 60 share the same epitope which comprises segments, $_{103}$NLTLLDSGL$_{111}$ (SEQ ID NO:62), and $_{136}$VQTGKDAPSNC$_{146}$ (SEQ ID NO:63) (Figure 28 top). Anti-VISTA VSTB95 and 112 appear to target similar epitopes, comprising segments $_{27}$PVDKGHDVTF$_{36}$ (SEQ ID NO:74), and $_{54}$RRPIRNLTFQDL$_{65}$ (SEQ ID NO:65) (Figure 28 bottom). There are two other segments showing weak perturbation by VSTB95 and 112, including residues 39-52 and 118-134. However, the levels of the reduction are not as strong as the previous regions (27-36 and 54-65) in the differential map. Although one peptide, $_{100}$TMR$_{102}$ showing strong perturbation by VSTB95 and 112, is located on the other face of VISTA surface, it is distant from the epitope regions, 27-36 and 54-65. This perturbation could be due to allosteric effect. These HDX-MS results provide the peptide level epitopes for anti-VISTA antibodies. There were no overlapping epitope regions for these two epitope groups. These results are in agreement with the previous competition binning data in that they do not compete with each other.

[00309] EXAMPLE 14: STRUCTURE DETERMINATION OF THE HUMAN VISTA ECD:VSTB112 FAB COMPLEX BY PROTEIN CRYSTALLOGRAPHY

[00310] In an effort to determine the VISTA structure and to delineate the epitope and paratope defining the interaction between VISTA extracellular domain (ECD) and the Fab fragment of lead antibody VSTB112, the complex was crystallized and structure determined to 1.85 Å resolution. The structure of the ECD of human VISTA in complex with the Fab fragment of the antibody VSTB112 was determined in an effort both to determine the structure of VISTA ECD itself and to define the epitope/paratope for this interaction. The structure reveals VISTA to adopt an IgV fold with a chain topology similar to the TCR Vα chain. In addition to the canonical disulfide bond bridging B and F strands in the back and front faces of the β-sandwich, the structure reveals the ECD to have two additional disulfide bonds, one tethering the CC' loop to the front sheet and a second between the A' and G' strands. Although crystal contacts between VISTA molecules are present, they are minor and there is no evidence for a dimer of VISTA ECDs based on this structure. The VSTB112 epitope is shown to comprise the portions of the VISTA BC, CC', and FG loops together with residues of the front beta sheet (C'CFG) nearest those loops. The paratope is biased largely toward heavy chain interactions with CDR L3 making minimal contact.

[00311] Epitope/paratope defining VISTA:VSTB112 interaction

[00312] VSTB112 Fab buries a surface area of 1024.3 Å2 upon binding VISTA ECD, with burial of the heavy chain surface accounting for 715.3 Å2 of this total. Seven hydrogen bonds and 4 salt bridge interactions are formed between VISTA and VSTB112 light chain and 10 hydrogens and 2 salt bridge interactions between VISTA and VSTB112 heavy chain. VSTB112 recognizes residues in the front sheet strands C', C, F, and G on the ends proximal to the FG loop as well as residues in the BC, FG, and CC' loops (Figures 29 and 30). Interactions with the CC' loop account for most of the contacts with the Fab light chain with only residues E125 and R127 in the FG loop making additional light chain interactions. Residues 119 to 127 corresponding to the VISTA FG loop account for 38% of the total 1034.8 Å2 of surface area buried upon binding VSTB112. Notably, this loop is highly polar, comprised of the following sequence –IRHHHSEHR- (SEQ ID NO:75). Additionally, W103 in the VSTB112 CDR H3 packs nicely against the backbone of VISTA residues H122 and H123, and VISTA H121 makes an edge on interaction with the aromatic ring of F55 in CDR H2.

[00313] A comparison of epitope regions identified by crystallography and HDX is shown in Figure 31.

[00314] EXAMPLE 15: ACTIVATION OF T CELLS AND MONOCYTES BY ANTI-VISTA ANTIBODIES

[00315] The functional effect of anti-VISTA antibodies was evaluated in two *in vitro* assays, mixed leukocyte reaction (MLR) and SEB (*Staphylococcus* enterotoxin B). Both assays measure T cell proliferation and cytokine induction as their primary readouts, but these effects are due to different mechanisms. In the MLR, peripheral blood mononuclear cells (PBMCs) from two different human donors are incubated together, and major histocompatibility complex (MHC) mismatch between T cells of one donor and dendritic cells of the other donor results in T cell proliferation and interferon (IFNγ) production. In the SEB assay, PBMCs from a single donor are incubated with a bacterial superantigen, which directly links MHC Class II protein on the surface of antigen-presenting cells (APC) to the T-cell receptor (TCR) on T cells, causing T cell activation, proliferation, and cytokine secretion. In both assays, VSTB112, which is the parent molecule of VSTB174, demonstrated dose-dependent induction of T cell proliferation and cytokine production, and was most potent among the candidates (Figures 21A-21D, Table 12).

[00316] Table 12. EC50 values for the MLR assay readouts. VSTB112 (parent of VSTB174) was the most potent molecule.

Candidate	EC_{50} proliferation (μg/ml)	EC_{50} IFNγ production (μg/ml)
VSTB112	0.21	0.38
VSTB116	0.17	0.69
VSTB95	0.29	1.67
VSTB50	0.77	1.14
VSTB53	0.47	1.88
VSTB60	1.04	2.48

[00317] Monocyte Activation Assays

[00318] The assay data, shown in Table 12, was generated with VSTB112, the parent molecule of VSTB174. To better understand the activity of VSTB174, monocyte activation assays were conducted. The results showed that incubation of VSTB174 with whole PBMCs induced upregulation of activation markers (CD80 and HLA-DR) on CD14+ monocytes, indicating an effect of antibody binding to an immune cell subset known to expres high levels of VISTA (Figure 32). A further question is whether the effects on monocyte activation in whole PBMC could be facilitated by any antibody that binds VISTA and has an IgG1 Fc. Antibodies VSTB103 and VSTB63 bind to VISTA with high affinity (KD 6.36E-10 and 8.30E-10 respectively) and to cells expressing VISTA protein, similar to VSTB112 and VSTB111. VSTB103 is in the same epitope bin as VSTB112, while VSTB63 is in a different epitope bin; neither antibody facilitated monocyte activation. Taken together, these results show that one mechanism by which VSTB174 may exert its effect on T cell activation/proliferation is via monocyte activation facilitated by NK cells.

[00319] Preparation of Media

[00320] 500 ml of RPMI 1640 (Corning, 10-040-CV) was combined with 50 ml of human AB serum (Valley Biomedical, Inc, Lot # 3C0405), 5 ml of Penicillin/Streptomycin (Lonza, 17-602E) 10,000 U/ml, 5 ml of L-glutamine (100x) (Gibco, 25030-081) and 10 ml of HEPES (1M) (Fisher BP299-100, Lot#-1). Media was stored for no longer than 14 days at 4°C.

[00321] Preparation of soluble VISTA and control antibodies

[00322] Antibodies were diluted to 2X desired concentration in 10% AB serum media:
VSTB174: lot VSTB174.003

[00323] Added 100 μl of the appropriate antibody solutions to the appropriate wells of a 96 well U-bottom plate (Falcon, 353077). After the various cellular populations were added

in 100 µl, the final concentration of each antibody was 1, 0.1 or 0.01 g/ml. IgG1 control antibody CNTO 3930 (Lot 6405, ENDO <0.1 EU/mg) was added at a final concentration of 1 µg/ml.

[00324] The PBMCs were isolated

[00325] Donors were at least 18 years of age, generally healthy and selected randomly from the local population.

[00326] Donor blood was transferred from isolation tube to 50 ml conicals.

[00327] 15 mls of Ficoll 1077 (SIGMA, 10771) were under-laid being careful not to mix with the blood. This was per 25 mls of blood.

[00328] The cells were centrifuged at 1250g for 25 minutes at room temperature with no brake.

[00329] The white blood cells were isolated at the interphase of the Ficoll and the serum and the cells were diluted into 40 ml of Hanks Balanced Salt Solution (HBSS).

[00330] The cells were centrifuged at 453g (1500 rpm) for 10 minutes at 4 C.

[00331] The cells were resuspended in 50 mls of HBSS and were counted by transferring 500 l to a separate eppendorf tube.

[00332] Additionally, a Pan Monocyte isolation kit from Miltenyi was used per manufacturer's instructions (cat# 130-096-537) to isolate CD14+ cells by negative selection in several treatment groups.

[00333] *In vitro* culture setup

[00334] The appropriate number of cells needed was determined for the assay based on the number of samples to be analyzed. The responder population was seeded at 2.0x10^5cells/well of a 96-well U-bottom plate. For the CD14 negatively selected population, 0.5x10^5 cells were seeded. All conditions were performed in triplicate.

[00335] The cells were centrifuged as described above and resuspended at a concentration of 2x10^6/ml for the whole PBMC population and 0.5x10^6/ml for the CD14 negatively selected population in 10% AB serum media and added 100 l of test antibody to appropriate wells bringing the total volume in each well to 200 l.

[00336] The cells were incubated for 1, 2, or 3 days at 37° C and 5% CO$_2$.

[00337] Antibody staining and flow cytometry

[00338] The 96 well U-bottom plate was centrifuged for 5 minutes at 453g and removed the supernatant.

[00339] Cells were washed with 200 μl PBS and centrifuged as in step 5.5.1.

[00340] The supernatant was discarded and resuspended in 50 μl of PBS containing the following antibodies:

- CD14-APC (clone HCD14) 1:250 (Biolegend cat #325608)
- HLA-DR-PE Cy7 (clone L243) 1:250 (Biolegend cat # 307616)
- CD80-PE (clone 2D10) 1:250 (Biolegend cat # 305208)
- Hu FcR binding inhibitor (eBioscience cat # 14-9161-73)

[00341] Was incubated for 20 minutes on wet ice in the dark.

[00342] 150 μl of PBS was added and centrifuged as in step 5.5.1.

[00343] 150 l of PBS buffer was added and analyzed via FACS.

[00344] Samples were run on a Miltenyi MACSQuant 10-parameter flow cytometer and analyzed using FlowJo 9.7.5 for expression of HLA-DR and CD80 on the CD14+ population. Geometric mean fluorescence intensity (MFI), a statistic that defines the central tendency of a set of numbers, was used as the defining statistic to compare treatments.

[00345] Statistical Analysis

[00346] All statistics were carried out in Prism GraphPad, version 6. Pair-wise comparisons amongst the groups were made at each of the time-points using One-Way ANOVA with Tukey correction for multiplicity. P-values less than 0.05 for all tests and comparisons were deemed significant. For all graphs and tables, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$.

[00347] EXAMPLE 16: ADCC AND ADCP ACTIVITIES OF ANTI-VISTA ANTIBODIES

[00348] VSTB174 has an IgG1 Fc, which can confer antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) activity. Both types of assays were conducted and showed that VSTB174 could lyse or phagocytose K562-VISTA cells (Figures 33-34), but not K562 myeloma cell line parental cells (data not shown). An additional mechanism of action of VSTB174 to modulate the inhibitory action of VISTA may be the lysis or engulfment of cells expressing high levels of VISTA, thus removing them from the local microenvironment.

[00349] EXAMPLE 17: ADCP ACTIVITIES OF ADDITIONAL ANTI-VISTA ANTIBODIES

[00350] An *in vitro* phagocytosis assay was used to study the enhancement of macrophage-mediated phagocytosis of cells ectopically expressing VISTA by anti-human VISTA mAbs (VSTB173 and VSTB174). These mAbs were cloned into different Fc backbones (IgG1 WT (wild type), IgG1 PR (protease resistant), and IgG2σ) and were postulated to potentially have different activities with respect to enhancing phagocytosis. The IgG1 and IgG1 PR backbones are capable of binding to Fc receptors and have the potential to cause ADCP, while the IgG2σ does not bind to Fc receptors and should not mediate ADCP.

[00351] Anti-VISTA antibodies were tested in ADCP assays with K562 parental and K562-VISTA target cells. As shown in Figures 35-36, VSTB174, VSTB149, VSTB173 and VSTB145 enhanced hMac phagocytosis of K562-VISTA cells. VISTA antibodies VSTB140 or VSTB132, with the IgG2σ Fc that did not bind Fc receptors, did not enhance phagocytosis as expected. VISTA mAbs VSTB174 and VSTB173 with IgG1 Fc showed more robust phagocytosis than VSTB149 and VSTB145 with the IgG1PR Fc (see Tables 13 and 14 for EC_{50} values).

[00352] Table 13. Anti-human VISTA mAb EC_{50} values.

Treatment	VSTB174	VSTB149	VSTB140
EC_{50}	0.0782	0.1142	NA

[00353] Table 14. Anti-human VISTA mAb EC_{50} values.

Treatment	VSTB173	VSTB145	VSTB132
EC_{50}	0.0146	0.1075	NA

[00354] VSTB174 and VSTB173 showed weak enhancement of phagocytosis of K562 parental cells at the highest concentration (Figures 35-36), which may be due to low expression of VISTA by the K562 cells. The other anti-VISTA antibodies did not enhance phagocytosis of the K562 cells.

[00355] The negative control antibodies were each tested at two different concentrations in the K562-VISTA phagocytosis assay, but did not induce any phagocytosis. This result indicates that the phagocytosis mediated by the anti-VISTA antibodies is specific and due to VISTA antigen expression by the K562-VISTA cells.

[00356] EXAMPLE 18: ADCC ACTIVITIES OF ADDITIONAL ANTI-VISTA ANTIBODIES

[00357] In order to test their ability to induce ADCC, the following three human anti-VISTA antibodies were tested:

VSTB174 (IgG1)
VSTB149 (IgG1 PR)
VSTB174.LF (IgG1 LF (low fucose)).

[00358] Each antibody was tested at six different concentrations within the same plate, in triplicate over two separate experiments for a total of six data points.

[00359] VSTB174, VSTB149, and VSTB174.LF each demonstrated measurable ADCC activity at 10, 1, 0.1 and 0.01 µg/mL, while only the LF antibody demonstrated measurable ADCC activity at 0.001 µg/mL; none of the antibodies demonstrated ADCC at 0.0001 µg/mL. As each of these antibodies has an IgG1 or IgG1 variant Fc, this result is expected. The LF antibody demonstrated increased ADCC potency as evidenced by the smaller EC_{50} value for the LF antibody curve (0.002293µg/mL) as compared to the regular IgG1 antibody curve (0.02381µg/mL). The IgG1 PR antibody curve had an EC_{50} value similar to the regular IgG1 curve (0.01846µg/mL).

[00360] Table 15. EC_{50} values (µg/mL) of three tested anti-VISTA antibodies as determined by ADCC analysis.

anti-VISTA Antibody	EC_{50} (µg/mL)
VSTB174 (IgG1)	0.02381
VSTB149 (IgG1 PR)	0.01846
VSTB174.LF (IgG1 LF)	0.002293

[00361] The human IgG1, human IgG1 PR and human IgG1 LF antibodies all showed measurable ADCC mediated killing at the 10, 1, 0.1 and 0.01 µg/mL antibody concentrations, while only the LF antibody showed killing at the 0.001 µg/mL antibody concentration. None of the anti-VISTA antibodies showed killing at the 0.0001 µg/mL antibody concentration.

[00362] The LF antibody showed approximately 10 times more potent ADCC killing than either the regular IgG1 antibody or the IgG1 PR antibody, as seen in the EC50 values.

[00363] EXAMPLE 19: AFFINITY OF VSTB174 FOR HUMAN AND CYNOMOLGUS VISTA

[00364] The affinity of VSTB174 for human and cynomolgus monkey VISTA extracellular domain (ECD) was determined by surface plasmon resonance (SPR) methods on a ProteOn instrument. VSTB174 displayed very similar KD values for each protein, 1.56E-10 M for human VISTA ECD and 8.66E-11 M for cynomolgus VISTA.

[00365] EXAMPLE 20: VISTA ANTIBODIES EXHIBIT EFFICACY IN MURINE TUMOR MODELS

[00366] Mouse Strains, Reagents and Tumor Models

[00367] For the *in vivo* studies, human VISTA knockin (VISTA-KI) mice back-crossed onto a C57Bl/6 background were used.

[00368] An anti-human VISTA antibody was generated to enable testing in the VISTA-KI mice, using the VSTB174 variable region grafted onto mouse Fc IgG2a (VSTB123).

[00369] The MB49 bladder cancer was evaluated in the VISTA KI mice,

[00370] In addition to published studies demonstrating that anti-VISTA antibody therapy inhibits tumor growth in wild type mice (Le Mercier et al., 2014), anti-tumor efficacy has been demonstrated with the surrogate hamster antibody in wt mice using different dosing schedules, and in the VISTA-KI mice treated with VSTB123.

[00371] *In Vivo* Efficacy Studies in the MB49 Tumor Model in VISTA-KI Mice

[00372] MB49 efficacy studies were conducted in female VISTA-KI mice, testing VSTB123 at several doses ranging from 1- 10 mg/kg. Mice were injected intradermally with 250,000 MB49 tumor cells on day 0. On day 6, dosing began as indicated in Figure 37 (either 10 mg/kg of the isotype control mIgG2a, or the indicated doses of VSTB123; 10 mice/group).

[00373] VSTB123 was more effective at higher vs lower doses, as shown in Figure 37. Doses of 10 mg/kg and 7.5 mg/kg were equivalent, while tumors grew more quickly in the mice dosed at 5 or 1 mg/kg.

[00374] EXAMPLE 21: DETECTION OF VISTA EXPRESSION IN HUMAN TUMORS WITH ANTI-VISTA ANTIBODIES

Figure 1 shows VISTA expression by an AML tumor cell line—this and the RNA seq expression data in Figure 17 support the idea that VISTA is expressed by AML cells and that anti-VISTA drug be efficacious through directly targeting these cells for immune modulation or antibody-mediated killing.

[00375] Data to evaluate VISTA expression in lung cancer was obtained from lung tumor samples from surgical resections. Cells were dissociated and characterized for expression of VISTA and many other markers. Results showed that 13/13 lung tumors (squamous or adenocarcinomas) contained CD14+ VISTA+ myeloid cells, (Figure 38).

[00376] EXAMPLE 22: DETECTION OF VISTA EXPRESSION IN LUNG TUMORS USING ANTI-VISTA ANTIBODIES

[00377] An immunohistochemistry assay was optimized using clone GG8, an anti-human VISTA mouse IgG1. This mAb was used to investigate the staining of VISTA in non small cell lung cancer (NSCLC) FFPE tumor sections.

[00378] FFPE tumor sections were treated with standard antigen retrieval methods prior to staining. GG8 mouse anti-human VISTA antibody was used at a 1:500 dilution. GG8 binding was detected using a rabbit anti-mouse polyclonal antibody, followed by anti-rabbit polymer HRP. Counterstain with hematoxylin followed, then tumor sections were scored.

[00379] VISTA expression in lung cancer was mostly restricted to the immune infiltrate (example shown in Figure 39) and high levels of VISTA positive cells were present in many lung cancer samples

[00380] EXAMPLE 23: STRUCTURE OF THE EXTRACELLULAR DOMAIN (ECD) OF HUMAN VISTA IN COMPLEX WITH THE FAB FRAGMENT OF VSTB174

[00381] VISTA antigen variants were generated and purified for crystallography. Recombinant his-tagged VSTB174 Fab was internally expressed and purified. Crystals were generated and used to collect higher resolution data for the VISTA ECD:VSTB174 Fab complex using synchrotron radiation and the structural determination was solved using combinations of homology modeling and electron density analyses (Figure 29(Top)).

[00382] The structure of the VISTA ECD:VSTB174 Fab complex was determined by x-ray crystallography to a resolution of 1.85Å, providing the first structure of the VISTA ECD and delineating the VSTB174 epitope and paratope. The VISTA ECD adopts an IgV fold with a topology similar to CTLA-4 ECD, but possesses a unique G' strand that extends the front sheet of the β-sandwich. A' and G' are further tethered chemically via a disulfide bridge formed between residues C12 in the A' strand and C146 in the G' strand. Six cysteines were found to be engaged in three intramolecular disulfide bonds, and, based on crystal contacts, there is no evidence for a dimeric VISTA.

[00383] VSTB174 recognizes residues in the front sheet strands C', C, F, and G on the ends proximal to the FG loop as well as residues in the BC, FG, and CC' loops.

[00384] EXAMPLE 24: CLONING OF VSTB116 VH AND VL REGIONS INTO MOUSE IgG1 BACKBONE

[00385] The VSTB116 heavy chain (SEQ ID NO:57) and light chain (SEQ ID NO:58) variable regions were subcloned into a mouse IgG1 backbone to create VSTB175. The VSTB175 heavy chain expression plasmid, pDR23170, was generated using vector vDR000367 (pUnder vector encoding mouse IgG1 Balb C constant regions) and the VSTB116 variable region from pDR17582. Standard synthesis of pDR23170 was performed at Genewiz with HindIII_BamH1 sites and custom cloned into vector vDR000367 (pUnder_ mG1 Balb C) at HindIII _BamH1 sites making the final product of pDR23170. The VSTB175 light chain expression plasmid, pDR21003, was generated by standard synthesis of the variable region at GeneArt, with the addition of adapters. vDR000371 (pUnder vector encoding murine kappa constant region) was linearized using Esp3I sites. The synthesized fragment of VSTB175 light chain variable region sequence was then infusion cloned into vDR000371, creating pDR21003. The resultant primary transcripts were sequenced confirmed and large scale DNA preparations were prepared for transfections.

[00386] Table 16: Complete light chain and heavy chain sequences for VSTB175:

Protein ID	Heavy chain	Light chain
VSTB175*	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS*SYAIS*WVRQAPGQGLEWMG*GIIPIFGTANYAQKFQG*RVTITADESTSTAYMELSSLRSEDTAVYYCAR*SSYGWSYEFDY*WGQGTLVTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDLYTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMNTNGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGK (SEQ ID NO:76)	DIQMTQSPSSLSASVGDRVTITC*RASQSINTNLN*WYQQKPGKAPKLLIY*AASSLQS*GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC*QQARDTPIT*FGQGTKVEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO:77)

* Constant region sequences are underlined; putative CDR sequences are italicized.

[00387] EXAMPLE 25: EXPRESSION, PURIFICATION AND ANALYSIS OF VSTB175

[00388] Expi293F cells (Life Technologies Corporation Cat #A14527) were grown at 37°C; 7% CO2; 130 RPM in Expi293 Expression Medium (Life Technologies Corporation Cat # A14351-01). Two days prior to transfection, cells were split at 7e5 cells/ml. At the time of transfection, cells were counted and verified to be at a concentration of at least 30e5 cells/ml and above 95% viable. For each 30-mL transfection; 30 ug of plasmid DNA was mixed with in Opti-MEM I Reduced Serum Medium (Life Technologies Corporation Cat # 31985-070) to a total volume of 1.5 mL. (15ug of pAdvantage DNA and 15ug of expression vector DNA (for antibodies this is 1:3 ratio of HC:LC expression constructs). 81 uL of ExpiFectamine 293 Reagent (Life Technologies Corporation Cat # A14525). was then diluted in Opti-MEM I medium to a total volume of 1.5 mL. The diluted DNA and ExpiFectamine solutions were then mixed gently and incubated for 5 minutes at room temperature. The diluted DNA was added to the diluted ExpiFectamine 293 Reagent, mixed gently, and incubated for 20 minutes at room temperature. After the incubation the mixture was then added to 25.5 ml of cells in a 125 ml shake flask. Immediately following transfection, 150 uL of ExpiFectamine 293 Transfection Enhancer 1 and 1.5 mL of ExpiFectamine 293 Transfection Enhancer 2 were added to each flask (Life Technologies Corporation Cat # A14525). 5 days post transfection, cells supernatant was harvested by centrifugation and clarified through a 0.2 micron filter.

[00389] Two VSTB175 transient 293Expi transfection harvests were pooled and buffered to 1xDPBS + 0.5M NaCl. The sample was purified using an AKTApurifier at 2 - 10 C. The sample was captured to MabSelect SuRe resin (20mL, 1.6 cm ID x 10 cm H). Unbound sample was washed off the resin with 1xDPBS + 0.5M NaCl. The mAb was eluted with 0.1M Na+ Acetate pH 3.5. Elution fractions (8-mL) were collected when the OD280 was > 25 mAU/2mm. The elution fractions were neutralized with 2-mL 2.5M Tris HCl pH 7.2 per fraction and were stored at 2 - 10 C pending further processing. The elution peak fractions were analyzed by OD280 and SE-HPLC to determine protein concentrations and % aggregate. Due to a high amount of aggregate observed in this analysis the sample required SEC polishing. The samples were concentrated and SEC fractionation was done using a Superdex 200 column (26/60 column, 2.5mL/min 1xDPBS, 4-mL fractions). The sample was analyzed by OD280 (protein concentration), LAL (endotoxin content), SE-HPLC (% monomeric mAb), and SDS-PAGE. The sample was 98.22% monomeric mAb by SE-HPLC analysis.

[00390] 1) Soak anti-Human Fc biosensors in transfection media (for example, Freestyle 293 exprsesion medium (Life Technologies) or CHO) for 10min. before running assay. Add 120 μl media to FIA 96 well plate. Place 96 well plate in biosensor tray and place tips into media in tray. 2) Dilute samples 1:10 in transfection media (or 1:25 for concentrated samples). 3) Transfer 120 µl of each neat and 1:10 diluted sample into a second 96w black FIA plate. 4) Standard Curve already loaded onto the Octet machine. 5) Add three standard samples (40, 20, and 10 µg/ml of standard control antibody) after all unknown samples have been added. Fill the remaining wells of that column with transfection media as a reference. 6) Run samples in ForteBio Octet according to manufacturer instructions.

[00391] EXAMPLE 26: VSTB175 MOLECULAR PATHOLOGY IMMUNOHISTOCHEMISTRY ASSAY DEVELOPMENT

[00392] Recommended concentration: 10µg/ml; Diluent: Dako common antibody

[00393] Retrieval method utilized: Freshly prepared Dako low pH retrieval solution in pressurized vessel (Retriever) for 20 minutes @ 121° C followed by a 40 minute cool down in retriever. Remove and cool down 15 min at RT.

[00394] Detection System: Anti Mouse Polymer Envision + system DAB (Dako).

[00395] Instrument: Biogenex i6000

[00396] Protocol steps: 1) Deparaffinize 4µm paraffin sections to de-ionized water. 2) Antigen retrieval. 3) Rinse in de-ionized water. 4) Peroxidase Block (3% H_2O_2) 15 min. 5) Rinse in Wash Buffer for minimum of 15min. 6) protein block (Dako, Carpinteria, California) for 60 min RT. 7) Primary antibody 60 min. @ room temperature. 8) Rinse in Wash Buffer five times. 8) Peroxidase labled polymer anti-mouse 30 min. 9) Rinse in Wash Buffer five times. 10) Working DAB for 5 min. 11) Rinse in de-ionized water three times . 12) Rinse in Wash Buffer five times. 13) Counter stain in Mayers hematoxylin for 5 min. 14) Rinse in Tap water and run up to solvent prior to applying a coverslip.

[00397] Results are shown in Figure 41 and Tables 17-18.

[00398] Table 17: Tumor Microenvironment Compartment

Sample	Region	Tissue Area [mm2]	VISTA+ cells/mm2 (0.1 Gate)	VISTA+ cells/mm2 (0.125 Gate)
312979A2	TME	93.0	102.2	34.5
312980A1	TME	46.0	118.5	41.2
1168370B	TME	96.8	245.8	88.2
CNT15G5	TME	18.3	308.8	100.0
1172984B	TME	42.9	749.9	262.5

CNT15EG	TME	19.8	990.7	339.1
CNT15D3	TME	28.6	1719.7	652.9
CNT15CD	TME	27.2	1898.6	717.4
CNT0RQ1	AllCells	39.0	3305.9	1257.9

[00399] Table 18: Tumor compartment

Sample	Region	Tissue Area [mm2]	VISTA+ cells/mm2 (0.1 Gate)	VISTA+ cells/mm2 (0.125 Gate)
312979A2	Tumor	93.0	8.3	2.4
312980A1	Tumor	46.0	22.2	7.4
CNT15EG	Tumor	19.8	60.8	19.1
CNT15G5	Tumor	18.3	82.4	25.4
CNT15CD	Tumor	27.2	87.8	28.2
1168370B	Tumor	96.8	107.3	38.0
CNT15D3	Tumor	28.6	127.0	43.4
1172984B	Tumor	42.9	545.4	175.7

[00400] EXAMPLE 27: VSTB175 ANTIBODY CLONES IN LUNG CANCER TISSUE

[00401] Materials and Methods:

[00402] VISTA VSTB175 antibody and NSCLC samples: Frozen aliquots of the VISTA VSTB175 antibody were provided for optimization in human NSCLC tissue samples. Nine formalin-fixed, paraffin-embedded (FFPE) samples were selected for evaluation, and unstained sections on glass slides were provided for immunohistochemical (IHC) staining once an optimized VISTA (VSTB175) IHC assay was identified.

[00403] IHC assay optimization: VISTA (VSTB175) IHC assay optimization was performed on tonsil and spleen tissue. Two antigen retrieval times (20 min and 40 min) were evaluated for sodium citrate at pH 6 for a range of antibody dilutions (1:500, 1:1000, and 1:2000). The presentation of staining in tonsil and spleen tissue was evaluated by a board-certified veterinary pathologist, and the optimal assay conditions were identified. The optimal IHC assay used antigen retrieval with sodium citrate at pH 6 for 40 min at 90° C (this temperature was chosen because of the high altitude location of the laboratory), a VISTA (VSTB175) antibody dilution of 1:500 (6.36 mg/ml starting concentration) for 60 min, and development with DAB for 10 min. The IHC assay was performed using a Leica Bond RX Autostainer according to the following protocol:

[00404] Detailed protocol (using Leica Bond RX or Max Autostainer):

[00405] 1. Slides are cut at 4 microns per usual sectioning protocol. Slides are allowed to air dry and are then baked for 60 minutes at 60°C. Slides are generally used for staining within 2 weeks of cutting.

[00406] 2. Place baked slides on the Leica slide racks, place covertiles on slides, and insert rack into Autostainer.

[00407] 3. Dilute Primary antibody to 1:500 with Leica Antibody Diluent (#AR9352).

[00408] Steps performed on Leica Bond RX/Max Autostainer:

[00409] 1. Bond Dewax Solution (#AR9222) applied three times at 72°C.

[00410] 2. 100% Ethanol applied three times

[00411] 3. Leica Bond Wash 1X Solution (#AR9590) applied three times.

[00412] 4. Leica ER 1 Epitope Retrieval Solution (#AR9961) incubates for 40 minutes at 90°C.

[00413] 5. Leica Bond Wash 1X Solution applied five times.

[00414] 6. Thermo Pierce Protein-Free Blocking Buffer (# 37584) incubates for five minutes.

[00415] 7. No rinse before Antibody Application.

[00416] 8. Antibody dilution incubates for 60 minutes.

[00417] 9. Leica Bond Wash 1X Solution applied three times.

[00418] 10. Post Primary Link (Leica Bond Polymer Refine Kit #DS9800) incubates for 8 minutes.

[00419] 11. Leica Bond Wash 1X Solution applied for 2 minutes three times.

[00420] 12. Polymer (Leica Bond Polymer Refine Kit #DS9800) incubates for 8 minutes.

[00421] 13. Leica Bond Wash 1X Solution applied for 2 minutes twice.

[00422] 14. DI water rinse.

[00423] 15. Peroxide Block (Leica BPR Kit) incubates for 10 minutes.

[00424] 16. Leica Bond Wash 1X Solution applied twice.

[00425] 17. DI water rinse.

[00426] 18. DAB chromagen/substrate (Leica BPR Kit) incubates for 10 minutes.

[00427] 19. Leica Bond Wash 1X Solution applied three times.

[00428] 20. Hematoxylin counterstain (Leica BPR Kit) incubates for 2 minutes.

[00429] 21. DI water rinse.

[00430] 22. Leica Bond Wash 1X Solution applied once.

[00431] 23. DI water rinse.

[00432] 24. Remove slides from Leica Bond RX/Max

[00433] Dehydration and coverslipping performed manually as follows:

[00434] 23. Two changes of 95% Ethanol (1 minute each)

[00435] 24. Two changes of 100% Ethanol (1 minute each)

[00436] 25. Two changes of Xylene (2 minutes each)

[00437] 26. Coverslip with Cytoseal mounting media.

[00438] Slide scanning and annotations: Upon completion of IHC staining, glass slides were digitized using Aperio model CS and XT scanners. Once each glass slide was digitized, annotations were utilized to identify regions of analysis (ROAs). The ROAs captured analyzable tumor tissue within each sample and the surrounding stromal tissue compartment. Unwanted tissue features such as necrosis, large areas of anthracosis (carbon pigment), and lymphoid follicles (such as non-tumor associated pre-existing lymphoid populations) were removed from analysis by annotations. Each ROA identified by the annotations was evaluated and approved by a pathologist prior to performing image analysis.

[00439] Image analysis: The number of VISTA positive cells/mm2 was identified as the primary endpoint for this study prior to initiation of the project. Tumor cell nests and associated infiltrating inflammatory cells were analyzed as the tumor tissue compartment. Stromal cell regions, and associated infiltrating inflammatory cells, were analyzed as the tumor microenvironment (TME) tissue compartment. Results:

[00440] Results are shown in Tables 17 and 18 above.

[00441] IHC Assay Development for VISTA (VSTB175): A matrix of IHC assay conditions was tested to identify an optimal IHC assay for the VISTA VSTB175 antibody reagent. Table 19 outlines the conditions evaluated during optimization. Figure 42 demonstrates staining of a human tonsil section (positive control tissue) for two independent days of staining. Optimal assay conditions were identified by evaluating the tonsil (positive control) and spleen (negative control) tissue sections. Generally, few positive cells were

identified in tonsil follicles. Additionally, VISTA staining was observed in the sub-epithelia regions outside of follicles and was difficult to interpret.

[00442] Table 19: Assay Conditions Evaluated for Optimization

		VISTA Antibody Dilution		
		1:500	1:1000	1:2000
Antigen Retrieval Time (@ pH 6)	20 minutes	Sub-optimal conditions	Sub-optimal conditions	Sub-optimal conditions
	40 minutes	Optimal conditions	Sub-optimal conditions	Sub-optimal conditions

[00443] Once optimal assay conditions were identified, the nine human lung cancer samples were stained with the VISTA (VSTB175) IHC assay. Figure 43 shows three examples of VISTA+ (VSTB175) staining observed in the study samples. Initial subjective assessments of each study sample suggested very few, if any, VISTA+ cells. Staining was observed in a number of cells in each study sample.

[00444] VISTA Analysis Solution Development: A range of sensitive DAB (i.e. VISTA) positivity gates were assessed to determine the optimal gate for identifying low levels of VISTA staining observed in cells while minimizing false positive cells. The rank-order of samples was poorly preserved between both antibody clones, and suggested each antibody was capturing different phenomena in the tumor tissue compartment. These findings were consistent with the notion that the improved specificity of the VISTA VSTB175 clone led to fewer false positive cell detections in the tumor tissue compartment. Finally, the rank order of samples based on the VSTB175 clone was not dependent on the VISTA positivity gate, and further indicated that both gates were measuring the sample biologic information with different sensitivity.

[00445] Table 20: Rank-order comparisons of VISTA VSTB175; Rank-order of study samples based on VISTA+ cell density in the TME tissue compartment.

VISTA (VSTB175)			
Sample	Region	VISTA+ cells/mm2 (0.1 Gate)	VISTA+ cells/mm2 (0.125 Gate)
312979A2	TME	102.2	34.5

312980A1	TME	118.5	41.2
1168370B	TME	245.8	88.2
CNT15G5	TME	308.5	100.0
1172984B	TME	749.9	262.5
CNT15EG	TME	990.7	339.1
CNT15D3	TME	1719.7	652.9
CNT15CD	TME	1898.6	717.4
CNT0RQ1	AllCells	3305.9	1257.9

[00446] Table 21: Rank-order comparison of VISTA VSTB175; Rank-order for VISTA+ cell density in the tumor tissue compartment.

VISTA (VSTB175)			
Sample	Region	VISTA+ cells/mm2 (0.1 Gate)	VISTA+ cells/mm2 (0.125 Gate)
312979A2	Tumor	8.3	2.4
312980A1	Tumor	22.2	7.4
CNT15EG	Tumor	60.8	19.1
CNT15G5	Tumor	82.4	25.4
CNT15CD	Tumor	87.8	28.2
1168370B	Tumor	107.3	38.0
CNT15D3	Tumor	127.0	43.4
1172984B	Tumor	545.4	175.7

[00447] EXAMPLE 28: IMMUNOHISTOCHEMISTRY

[00448] Methods:

[00449] Antibody titration experiments were conducted with antibodies provided by Janssen. Immunohistochemical staining was carried out with VSTB175 (chimeric antibody) and one ready-to-use mouse IgG1 isotype control antibody (BioGenex catalog# HK119-7M, referred to as BGX-Ms-IgG1) to establish concentrations that would result in minimal background and maximal detection of signal. Serial dilutions were performed at 20 μg/ml, 10 μg/ml, 5 μg/ml, and 2.5 μg/ml with the two experimental antibodies on formalin-fixed, paraffin-embedded tissues supplied by LifeSpan and positive (Vista K562) and negative (Raji) control cell lines supplied by Janssen. The isotype control antibody was only used at the one ready-to-use concentration. Antibodies VSTB175 and BGX-Ms-IgG1 were used as the primary antibodies, and the principal detection system consisted of a Vector anti-mouse secondary (BA-2000), and a Vector ABC-AP kit (AK-5000) with a Vector Red substrate kit (SK-5100), which was used to produce a fuchsia-colored deposit.

[00450] Tissues were also stained with positive control antibodies (CD31 and vimentin) to ensure that the tissue antigens were preserved and accessible for immunohistochemical analysis. Only tissues that were positive for CD31 and vimentin staining were selected for the remainder of this study.

[00451] The negative control consisted of performing the entire immunohistochemistry procedure on adjacent sections in the absence of primary antibody, and these slides were appropriately negative.

[00452] Slides stained at the reported concentrations were imaged with a DVC 1310C digital camera coupled to a Nikon microscope. Images were stored as TIFF files with Adobe Photoshop. Results are shown in Figures 44-53.

[00453] [DELETED]

[00454] While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

[00455] In some aspects, embodiments of the present invention as described herein include the following items.

1. An isolated antibody, or antigen-binding fragment thereof, comprising an antigen-binding region that binds to a mammalian V-domain Ig Suppressor of T cell Activation (VISTA) protein, wherein the antibody comprises:
    a) an antibody VH domain comprising a VH CDR1 comprising the amino acid sequence of SEQ ID NO:31, a VH CDR2 comprising the amino acid sequence of SEQ ID NO:32 and a VH CDR3 comprising the amino acid sequence of SEQ ID NO:33;
    b) an antibody VL domain comprising a VL CDR1 comprising the amino acid sequence of SEQ ID NO:34, a VL CDR2 comprising the amino acid sequence

of SEQ ID NO:35 and a VL CDR3 comprising the amino acid sequence of SEQ ID NO:36;

c) a non-human antibody heavy chain constant region; and

d) a non-human antibody light chain constant region.

2. The antibody or antigen-binding fragment of Item 1, wherein the antibody VH domain comprises SEQ ID NO:64.

3. The antibody or antigen-binding fragment of Item 1 or 2, wherein the antibody VL domain comprises SEQ ID NO:45.

4. The antibody or antigen-binding fragment of any one of Items 1-3, wherein the non-human antibody heavy chain constant region is a murine antibody heavy chain constant region.

5. The antibody or antigen-binding fragment of Item 4, wherein the murine antibody heavy chain constant region is a murine IgG1 heavy chain constant region.

6. The antibody or antigen-binding fragment of Item 5, wherein the murine IgG1 heavy chain constant region comprises the heavy chain constant region in SEQ ID NO:76.

7. The antibody or antigen-binding fragment of Item 4, wherein the murine antibody heavy chain constant region is a murine IgG2a heavy chain constant region.

8. The antibody or antigen-binding fragment of any one of Items 1-7, wherein the non-human antibody light chain constant region is a murine antibody light chain constant region.

9. The antibody or antigen-binding fragment of Item 8, wherein the murine antibody light chain constant region is a murine IgG1 light chain constant region.

10. The antibody or antigen-binding fragment of Item 9, wherein the murine IgG1 light chain constant region comprises the light chain constant region in SEQ ID NO:77.

11. The antibody or antigen-binding fragment of Item 8, wherein the murine antibody light chain constant region is a murine IgG2a light chain constant region.

12. The antibody or antigen-binding fragment of any one of Items 1-11, wherein the mammalian VISTA protein is a human VISTA protein.

13. The antibody or antigen-binding fragment of any one of Items 1-12, wherein the antibody or antigen-binding fragment binds to an epitope in SEQ ID NO:46.

14. The antibody or antigen-binding fragment of any one of Items 1-13, wherein the antibody is a whole antibody.

15. An isolated antibody, or antigen-binding fragment thereof, comprising an antigen-binding region that binds to a mammalian V-domain Ig Suppressor of T cell Activation (VISTA) protein, wherein the antibody comprises an antibody heavy chain comprising SEQ ID NO:76 and an antibody light chain comprising SEQ ID NO:77.

16. A composition comprising the antibody or antigen-binding fragment of Item 1 or 15 and a diagnostically acceptable carrier.

17. A method for detecting a mammalian VISTA protein in a sample, comprising contacting the sample with the antibody or antigen-binding fragment of Item 1 or 15 under conditions in which the antibody or antigen-binding fragment binds to VISTA protein in the sample, and detecting the antibody or antigen-binding fragment that is bound to VISTA protein in the sample.

18. The method of Item 17, wherein the sample comprises cells.

19. The method of Item 17 or 18, wherein the sample comprises a tissue.

20. The method of Item 17, wherein the sample comprises immune cells or stromal cells, or a combination thereof.

21. The method of Item 20, wherein the immune cells are myeloid cells, monocytes or T cells.

22. The method of Item 18 or 19, wherein the cells or tissue in the sample comprises cancer cells.

23. The method of Item 22, wherein the cancer cells are selected from the group consisting of lung cancer cells, prostate cancer cells, acute myeloid leukemia (AML) cells, melanoma cells, ovarian cancer cells and colon cancer cells.

24. The method of any one of Items 17-23, wherein the antibody or antigen-binding fragment comprises a detectable label.

25. The method of any one of Items 17-24, wherein the detecting method comprises an immunohistochemical (IHC) staining assay.

26. The method of any one of Items 17-24, wherein the detecting method comprises a flow cytometry assay.

CLAIMS

What is claimed:

1. An isolated antibody, or antigen-binding fragment thereof, comprising an antigen-binding region that binds to a mammalian V-domain Ig Suppressor of T cell Activation (VISTA) protein, wherein the antibody comprises:
   a) an antibody VH domain comprising a VH CDR1 comprising the amino acid sequence of SEQ ID NO:31, a VH CDR2 comprising the amino acid sequence of SEQ ID NO:32 and a VH CDR3 comprising the amino acid sequence of SEQ ID NO:33;
   b) an antibody VL domain comprising a VL CDR1 comprising the amino acid sequence of SEQ ID NO:34, a VL CDR2 comprising the amino acid sequence of SEQ ID NO:35 and a VL CDR3 comprising the amino acid sequence of SEQ ID NO:36;
   c) a non-human antibody heavy chain constant region; and
   d) a non-human antibody light chain constant region.

2. The antibody or antigen-binding fragment of Claim 1, wherein the antibody VH domain comprises SEQ ID NO:64.

3. The antibody or antigen-binding fragment of Claim 1 or 2, wherein the antibody VL domain comprises SEQ ID NO:45.

4. The antibody or antigen-binding fragment of any one of Claim 1-3, wherein the non-human antibody heavy chain constant region is a murine antibody heavy chain constant region.

5. The antibody or antigen-binding fragment of Claim 4, wherein the murine antibody heavy chain constant region is a murine IgG1 heavy chain constant region.

6. The antibody or antigen-binding fragment of Claim 5, wherein the murine IgG1 heavy chain constant region comprises the heavy chain constant region in SEQ ID NO:76.

7. The antibody or antigen-binding fragment of Claim 4, wherein the murine antibody heavy chain constant region is a murine IgG2a heavy chain constant region.

8. The antibody or antigen-binding fragment of any one of Claims 1-7, wherein the non-human antibody light chain constant region is a murine antibody light chain constant region.

9. The antibody or antigen-binding fragment of Claim 8, wherein the murine antibody light chain constant region is a murine IgG1 light chain constant region.

10. The antibody or antigen-binding fragment of Claim 9, wherein the murine IgG1 light chain constant region comprises the light chain constant region in SEQ ID NO:77.

11. The antibody or antigen-binding fragment of Claim 8, wherein the murine antibody light chain constant region is a murine IgG2a light chain constant region.

12. The antibody or antigen-binding fragment of any one of Claims 1-11, wherein the mammalian VISTA protein is a human VISTA protein.

13. The antibody or antigen-binding fragment of any one of Claims 1-12, wherein the antibody or antigen-binding fragment binds to an epitope in SEQ ID NO:46.

14. The antibody or antigen-binding fragment of any one of Claims 1-13, wherein the antibody is a whole antibody.

15. An isolated antibody, or antigen-binding fragment thereof, comprising an antigen-binding region that binds to a mammalian V-domain Ig Suppressor of T cell Activation (VISTA) protein, wherein the antibody comprises an antibody heavy chain comprising SEQ ID NO:76 and an antibody light chain comprising SEQ ID NO:77.

16. A composition comprising the antibody or antigen-binding fragment of Claim 1 or 15 and a diagnostically acceptable carrier.

17. A method for detecting a mammalian VISTA protein in a sample, comprising contacting the sample with the antibody or antigen-binding fragment of Claim 1 or 15 under conditions in which the antibody or antigen-binding fragment binds to VISTA protein in the sample, and detecting the antibody or antigen-binding fragment that is bound to VISTA protein in the sample.

18. The method of Claim 17, wherein the sample comprises cells.

19. The method of Claim 17 or 18, wherein the sample comprises a tissue.

20. The method of Claim 17, wherein the sample comprises immune cells or stromal cells, or a combination thereof.

21. The method of Claim 20, wherein the immune cells are myeloid cells, monocytes or T cells.

22. The method of Claim 18 or 19, wherein the cells or tissue in the sample comprises cancer cells.

23. The method of Claim 22, wherein the cancer cells are selected from the group consisting of lung cancer cells, prostate cancer cells, acute myeloid leukemia (AML) cells, melanoma cells, ovarian cancer cells and colon cancer cells.

24. The method of any one of Claims 17-23, wherein the antibody or antigen-binding fragment comprises a detectable label.

25. The method of any one of Claims 17-24, wherein the detecting method comprises an immunohistochemical (IHC) staining assay.

26. The method of any one of Claims 17-24, wherein the detecting method comprises a flow cytometry assay.

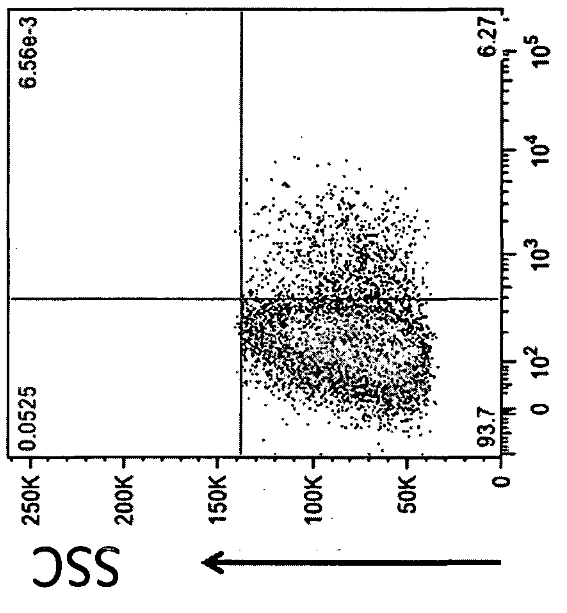


Figure 1A

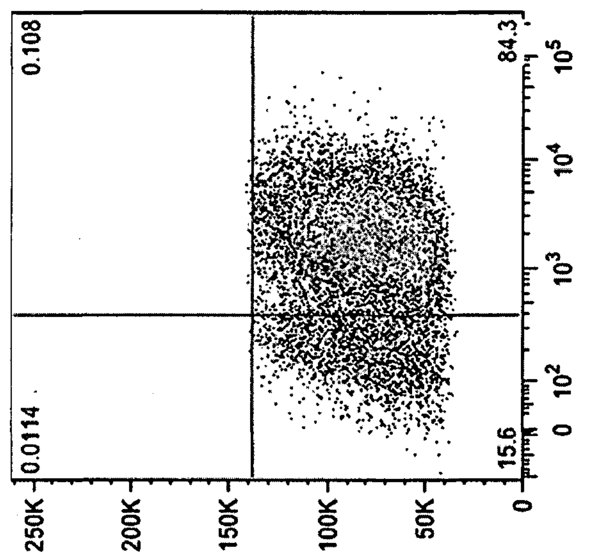


Figure 1B

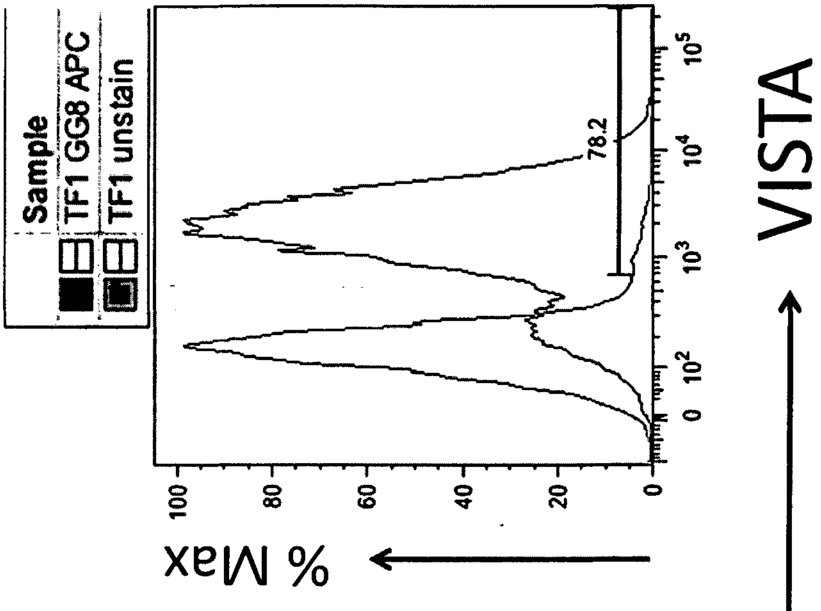


Figure 1C

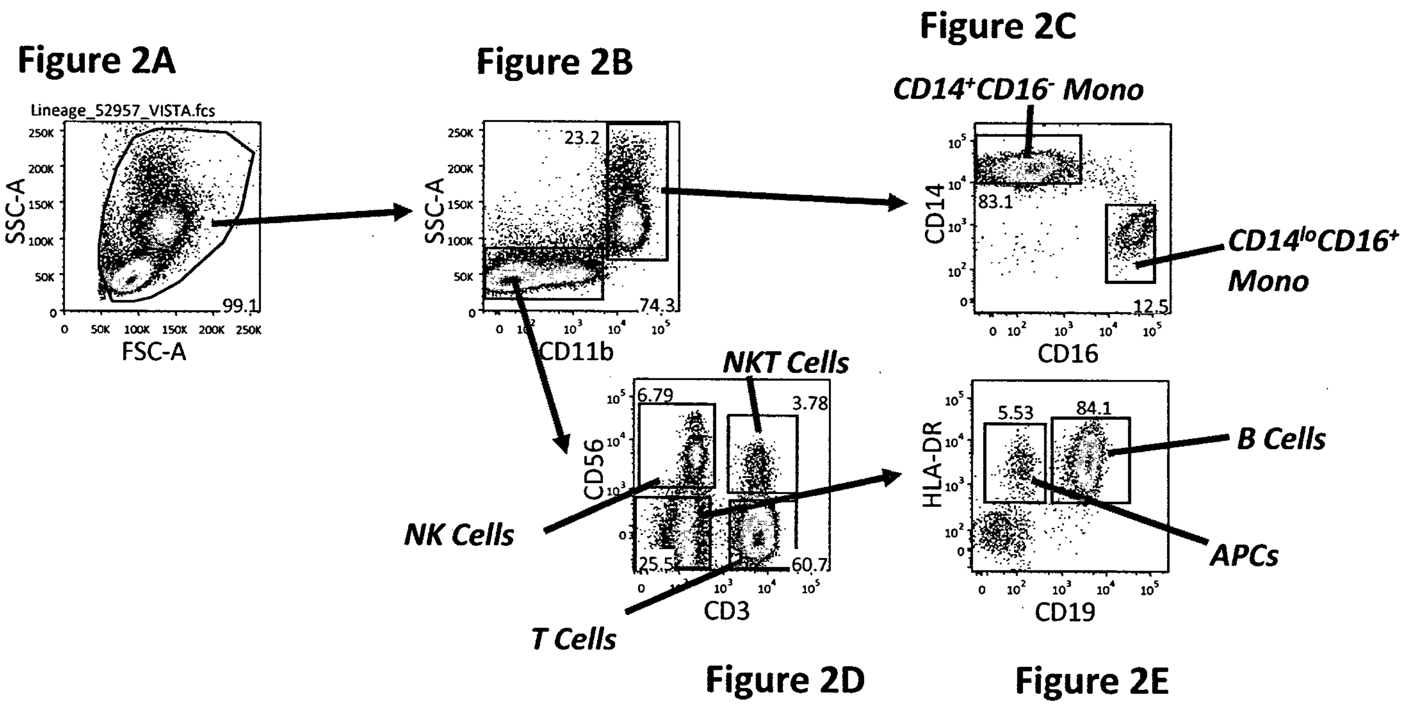


Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 2E

Figure 3A

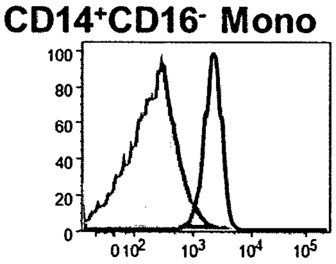


Figure 3B

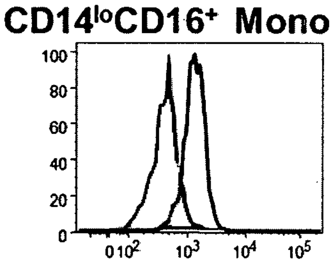


Figure 3C

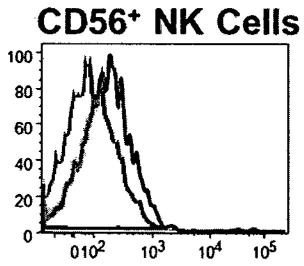
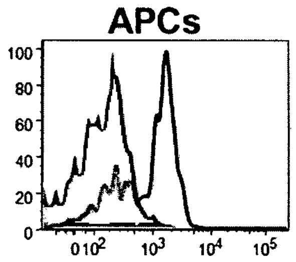


Figure 3D



☐ Isotype Control
— VISTA

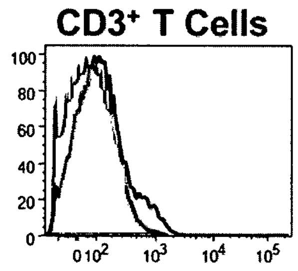


Figure 3E

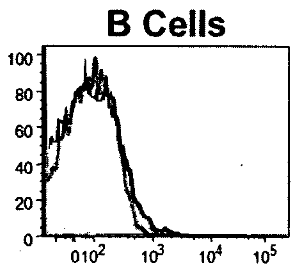


Figure 3F

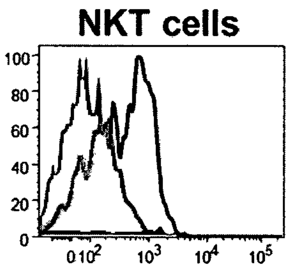


Figure 3G

VISTA →

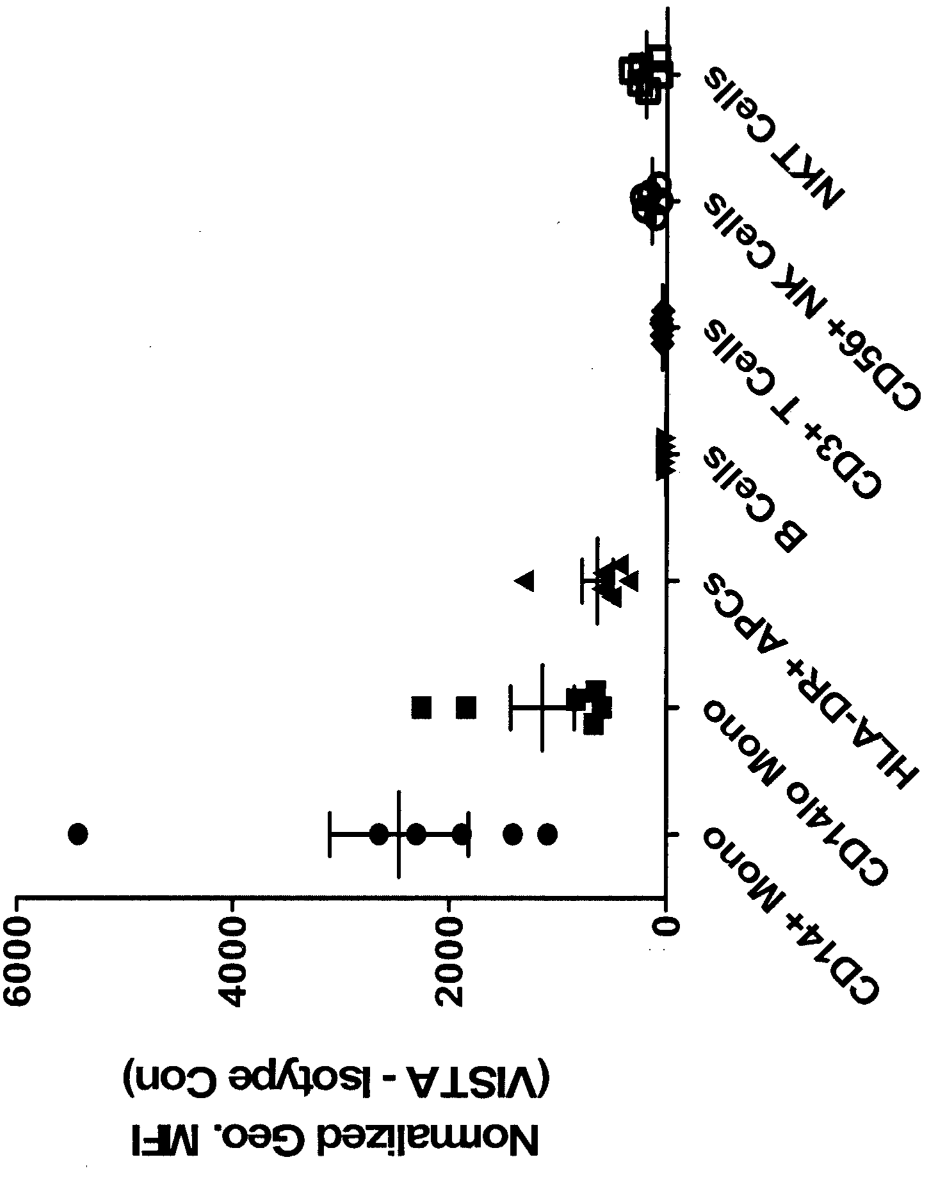

Normalized Geo. MFI
(VISTA - Isotype Con)
6000
4000
2000
0
CD14+ Mono
CD14lo Mono
HLA-DR+ APCs
B Cells
CD3+ T Cells
CD56+ NK Cells
NKT Cells

Figure 4

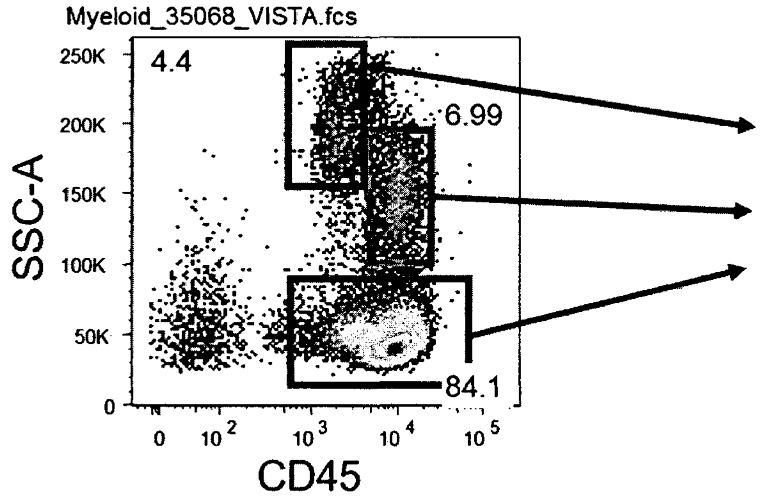


Figure 5A

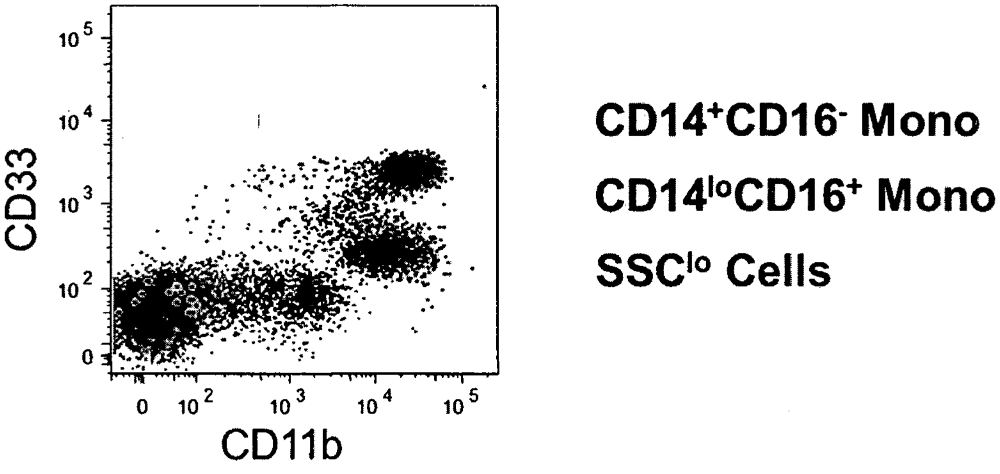


Figure 5B

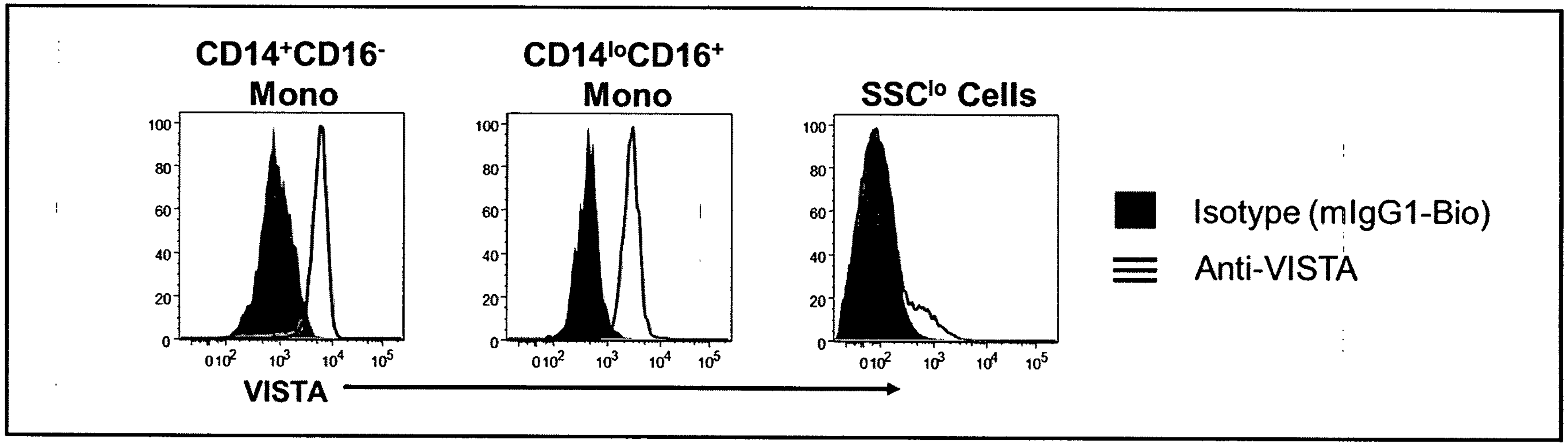


Figure 6A

Figure 6B

Figure 6C

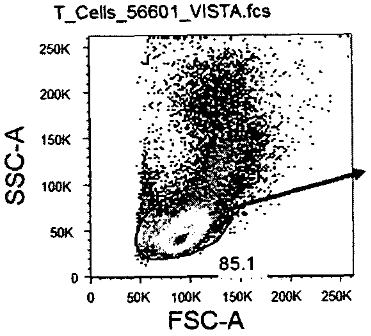


Figure 7A

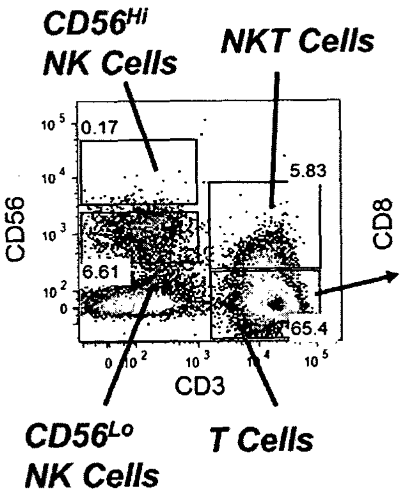


Figure 7B

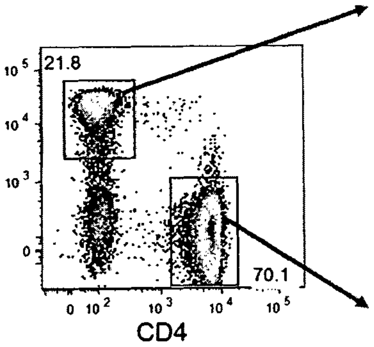
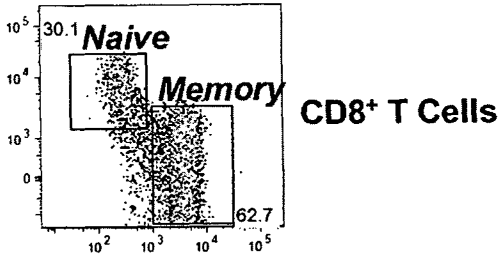


Figure 7C

Figure 7D



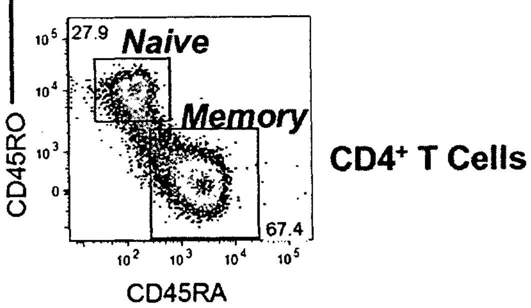


Figure 7E

Figure 8A Figure 8B Figure 8C

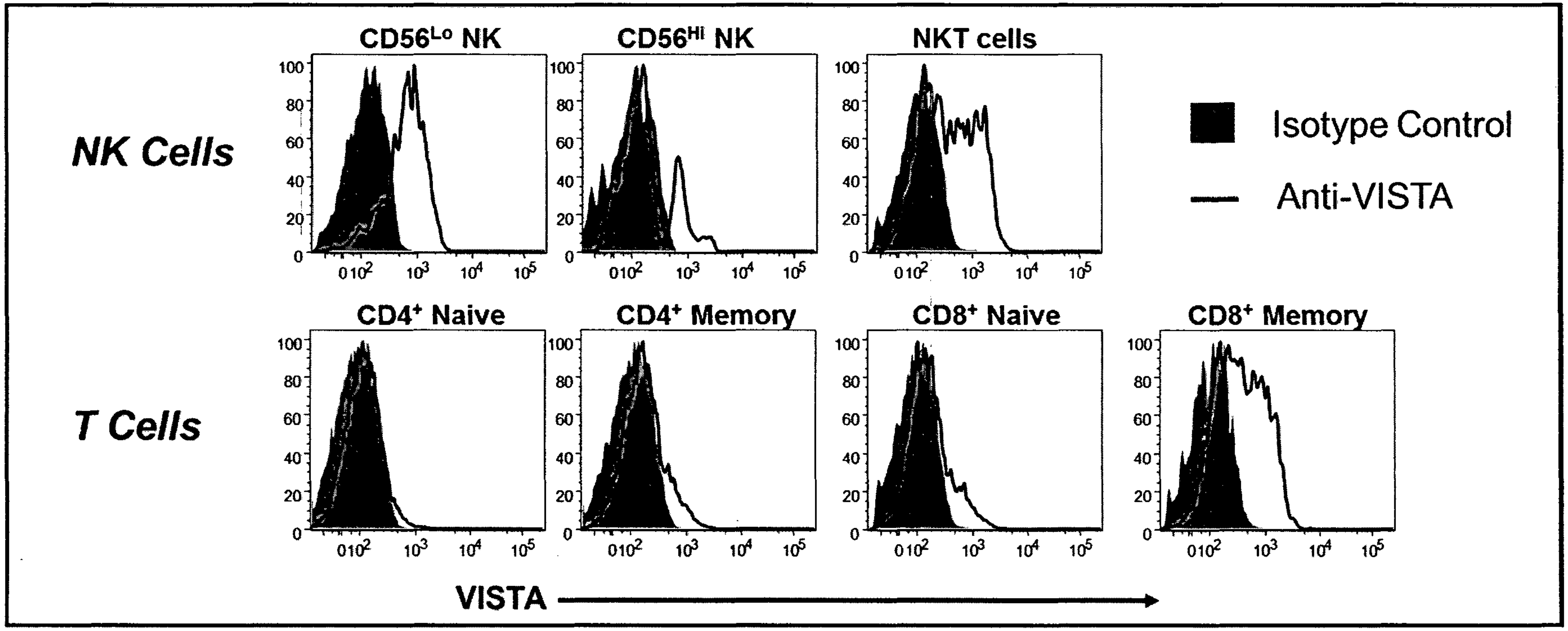


Figure 8D Figure 8E Figure 8F Figure 8G

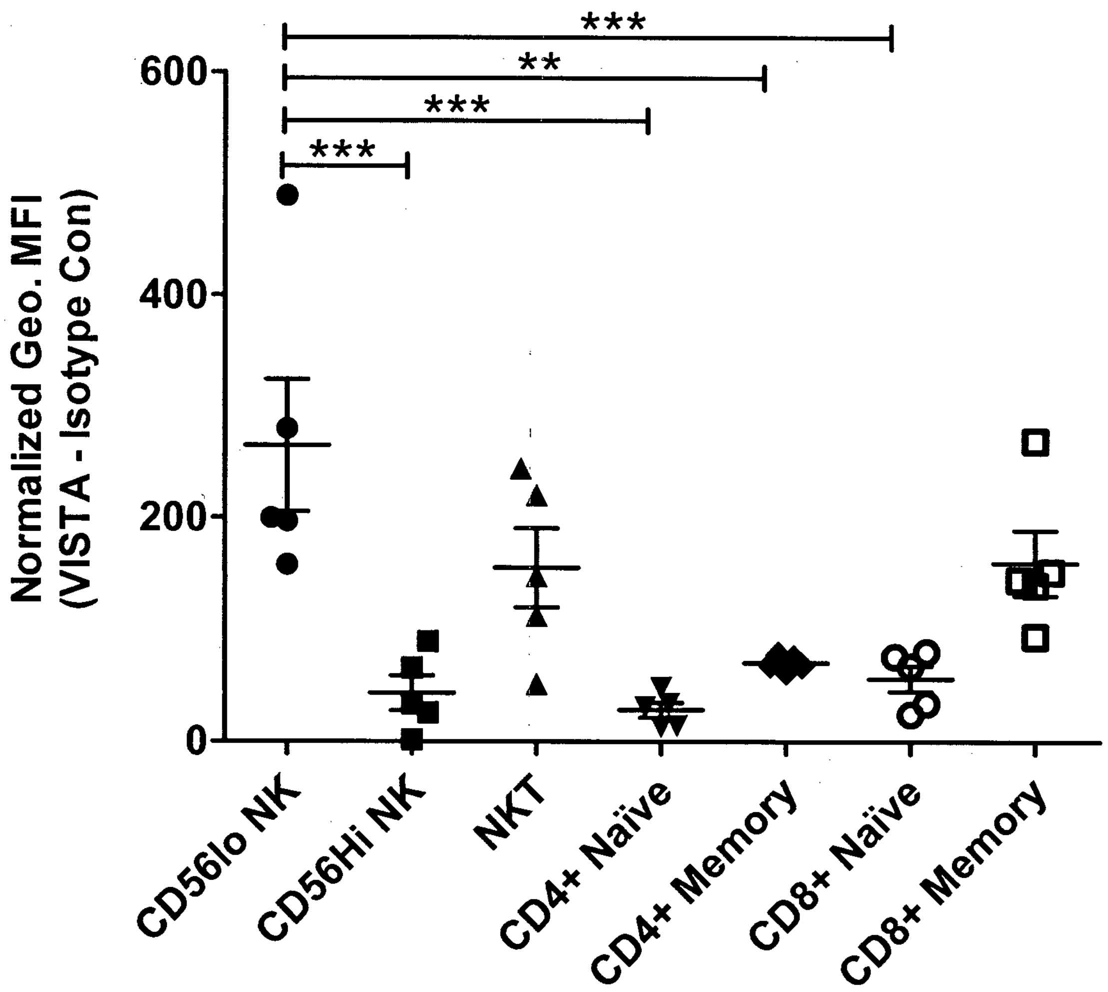

Normalized Geo. MFI
(VISTA - Isotype Con)
600
400
200
0
***
**
***
***
CD56lo NK
CD56Hi NK
NKT
CD4+ Naïve
CD4+ Memory
CD8+ Naïve
CD8+ Memory

Figure 9

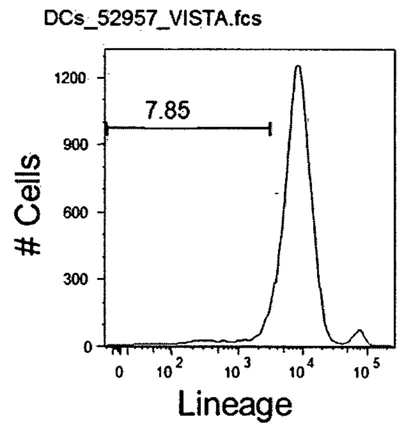


Figure 10A

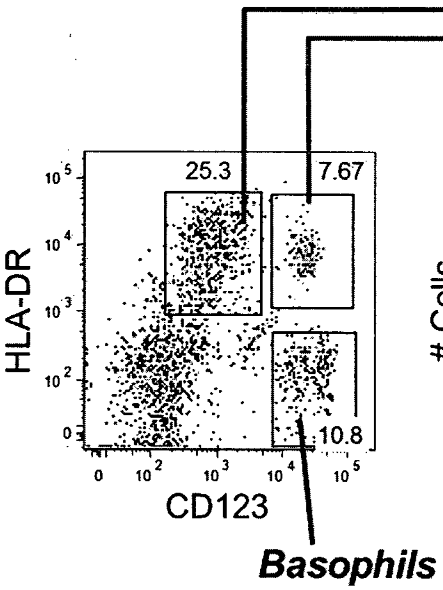


Figure 10B

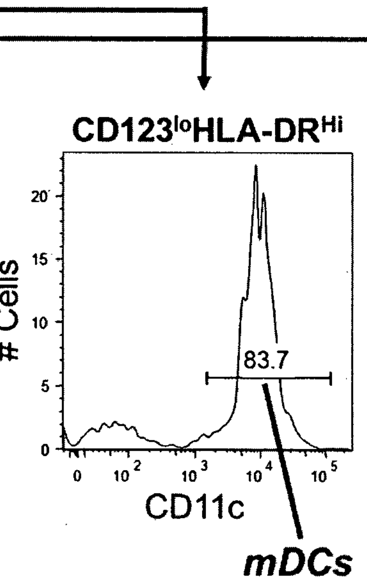


Figure 10C

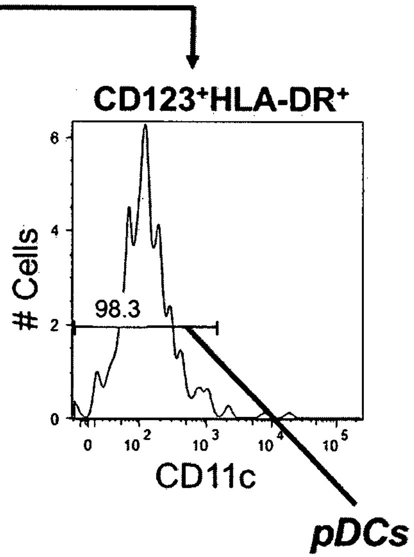


Figure 10D

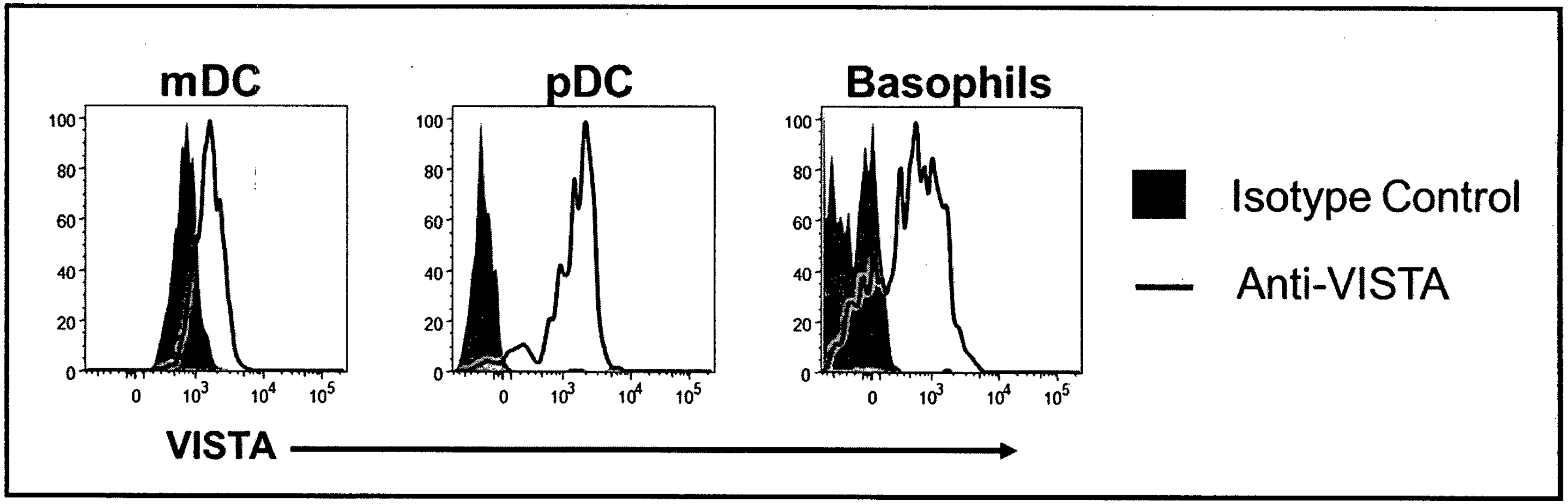


Figure 11A Figure 11B Figure 11C

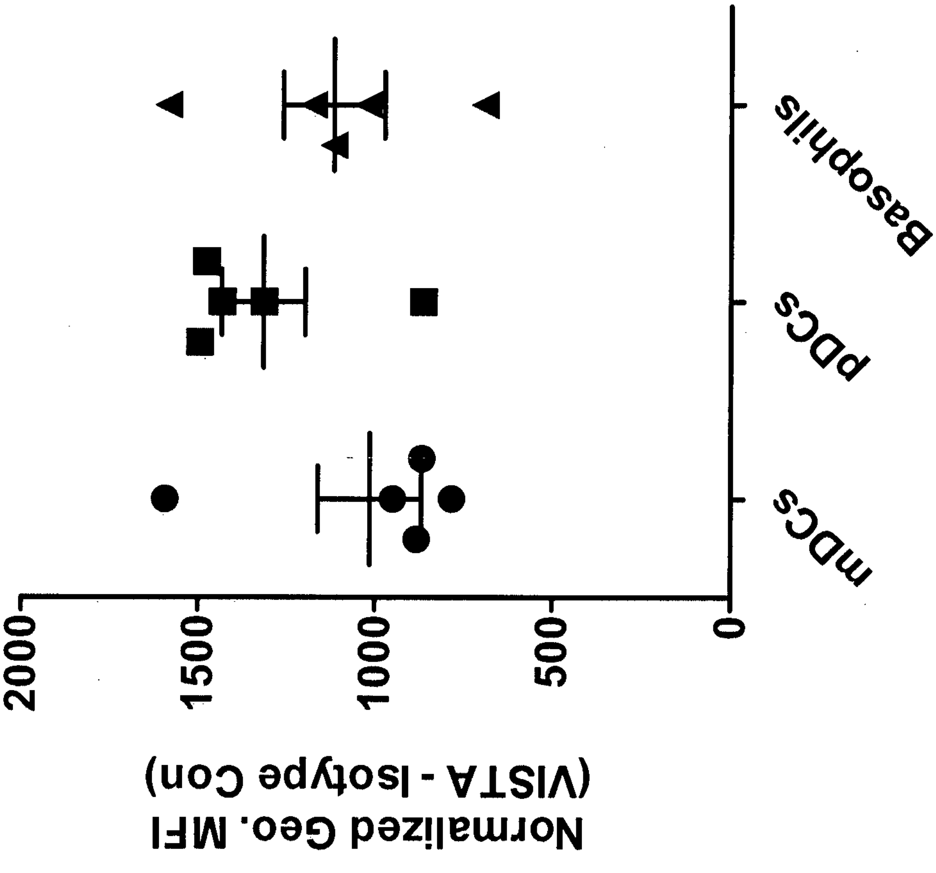

Normalized Geo. MFI
(VISTA - Isotype Con)
2000
1500
1000
500
0
mDCs
pDCs
Basophils

Figure 12

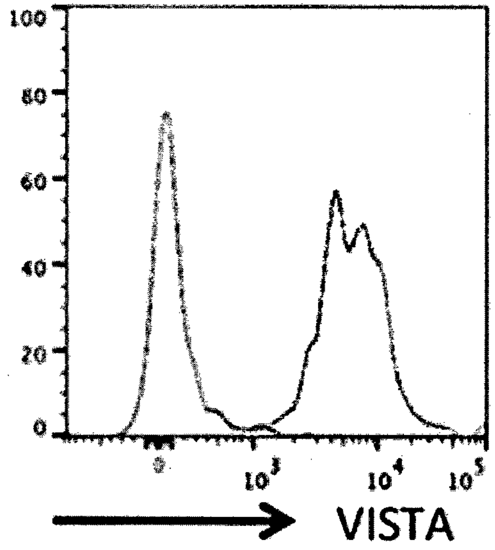


Figure 13A

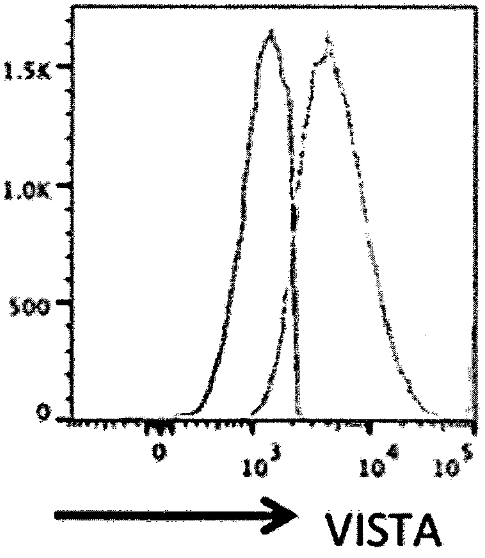


Figure 13B

Red – VISTA FMO
Blue – VISTA Staining

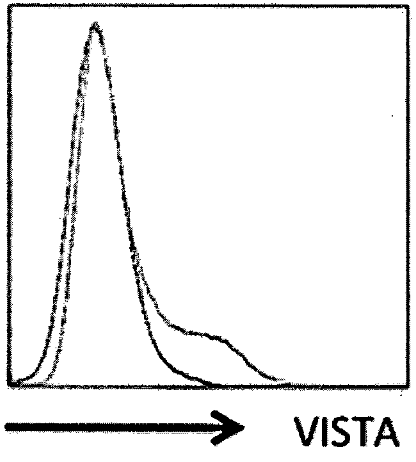


Figure 13C

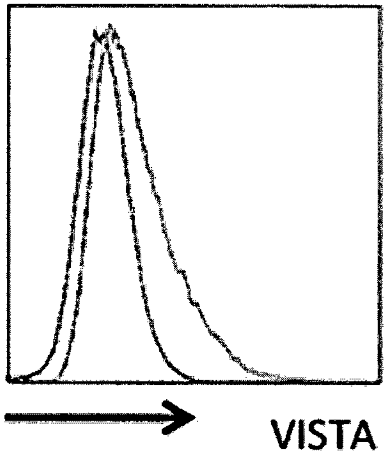


Figure 13D

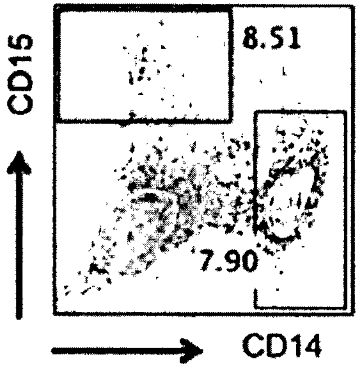


Figure 14A

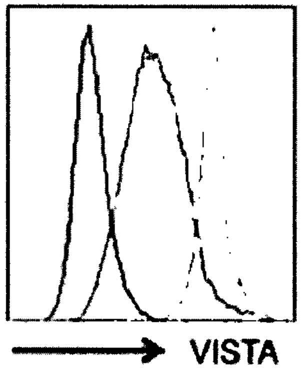


Figure 14B

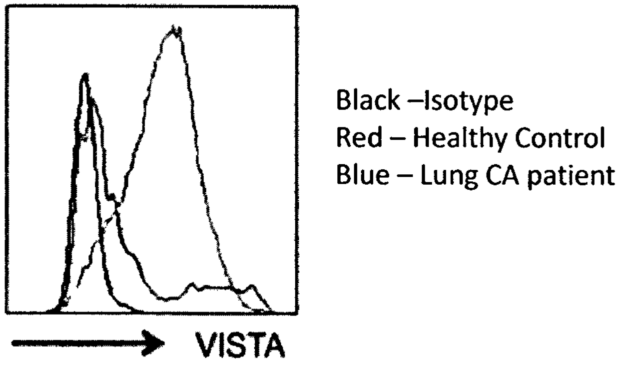


Figure 14C

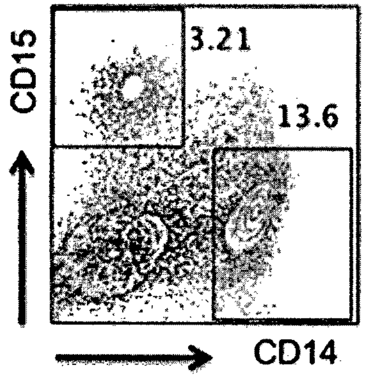


Figure 15A

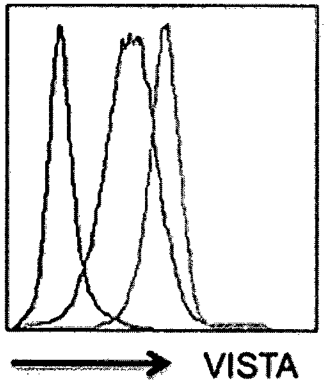


Figure 15B

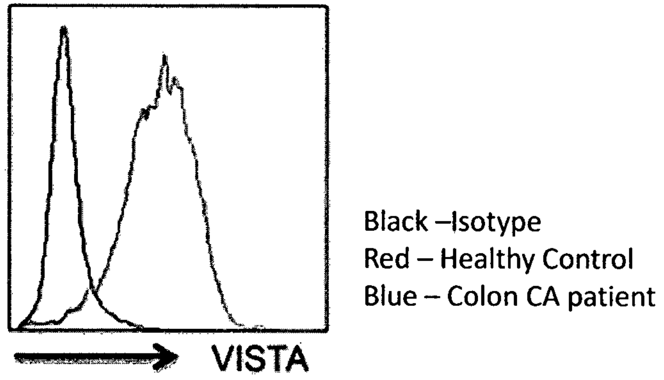


Figure 15C

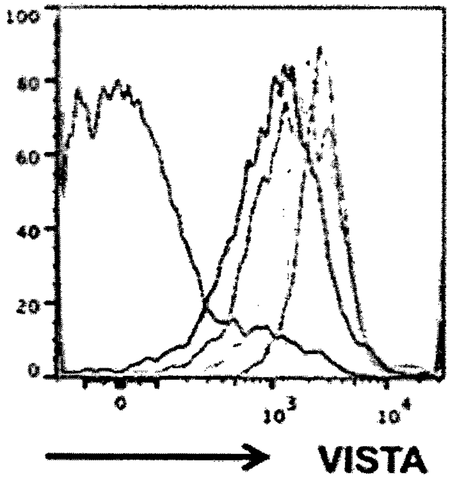


Figure 16A

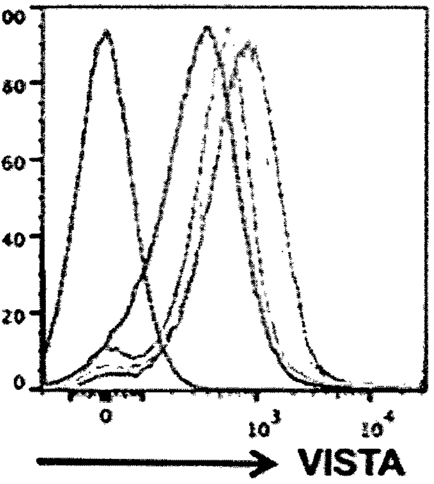


Figure 16B

Red – VISTA FMO
Blue – Monkey 1
Green- Monkey 2
Orange- Monkey 3
Black – Monkey 4

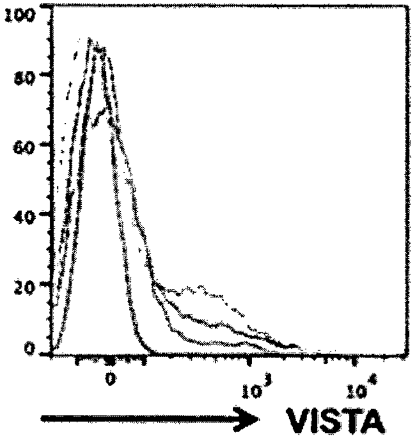


Figure 16C

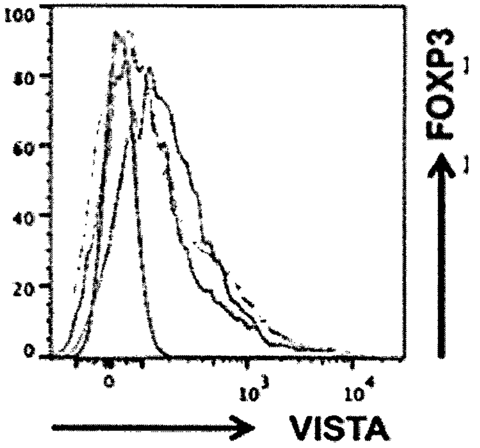


Figure 16D

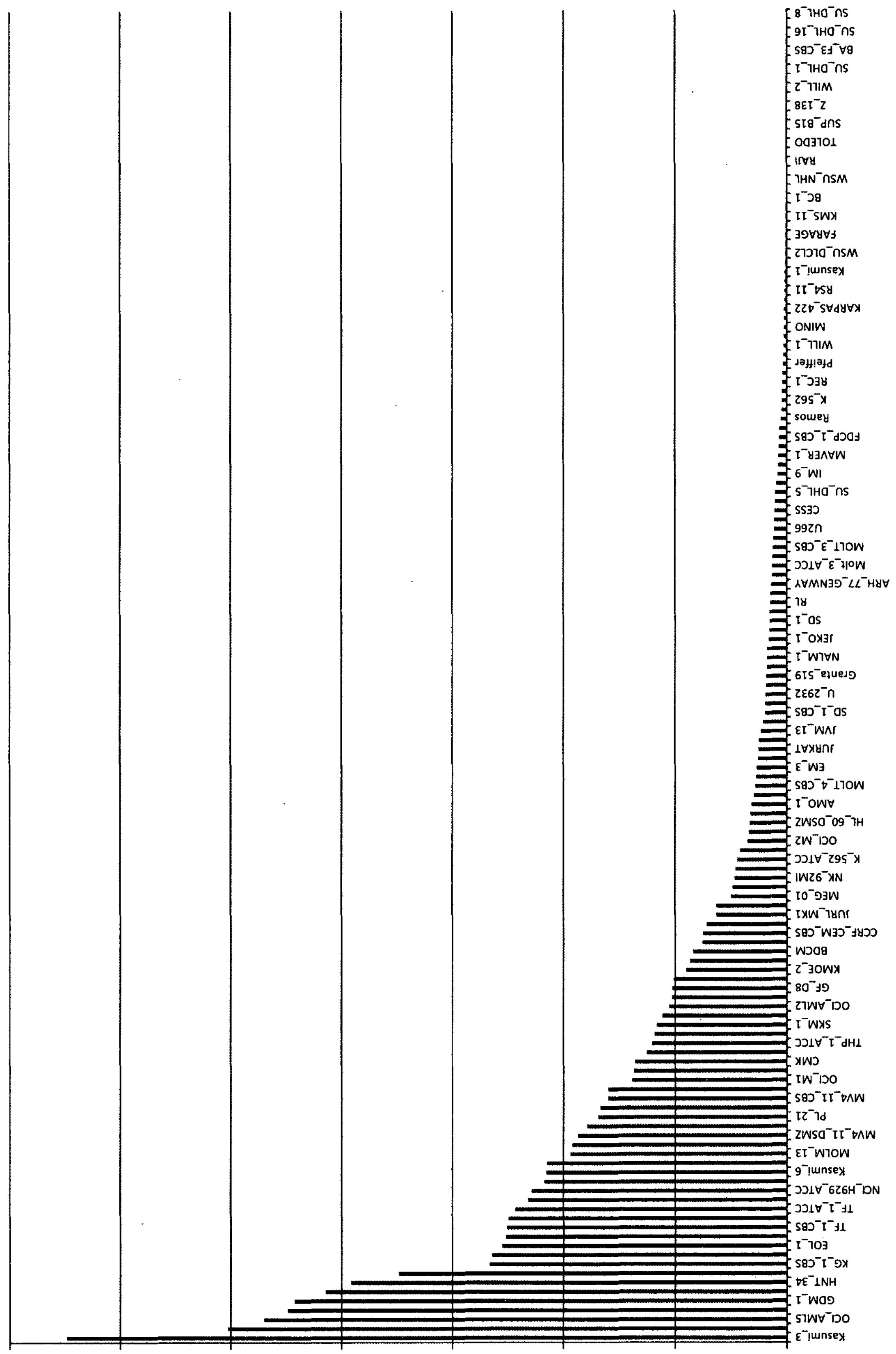

0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
Kasumi_3
OCI_AML5
GDM_1
HNT_34
KG_1_CBS
EOL_1
TF_1_CBS
TF_1_ATCC
NCI_H929_ATCC
Kasumi_6
MOLM_13
MV4_11_DSMZ
PL_21
MV4_11_CBS
OCI_M1
CMK
THP_1_ATCC
SKM_1
OCI_AML2
GF_D8
KMOE_2
BDCM
CCRF_CEM_CBS
JURL_MK1
MEG_01
NK_92MI
K_562_ATCC
OCI_M2
HL_60_DSMZ
AMO_1
MOLT_4_CBS
EM_3
JURKAT
JVM_13
SD_1_CBS
U_2932
Granta_519
NALM_1
JEKO_1
SD_1
RL
ARH_77_GENWAY
Molt_3_ATCC
MOLT_3_CBS
U266
CESS
SU_DHL_5
IM_9
MAVER_1
FDCP_1_CBS
Ramos
K_562
REC_1
Pfeiffer
WILL_1
MINO
KARPAS_422
RS4_11
Kasumi_1
WSU_DLCL2
FARAGE
KMS_11
BC_1
WSU_NHL
RAJI
TOLEDO
SUP_B15
Z_138
WILL_2
SU_DHL_1
BA_F3_CBS
SU_DHL_16
SU_DHL_8

Figure 17

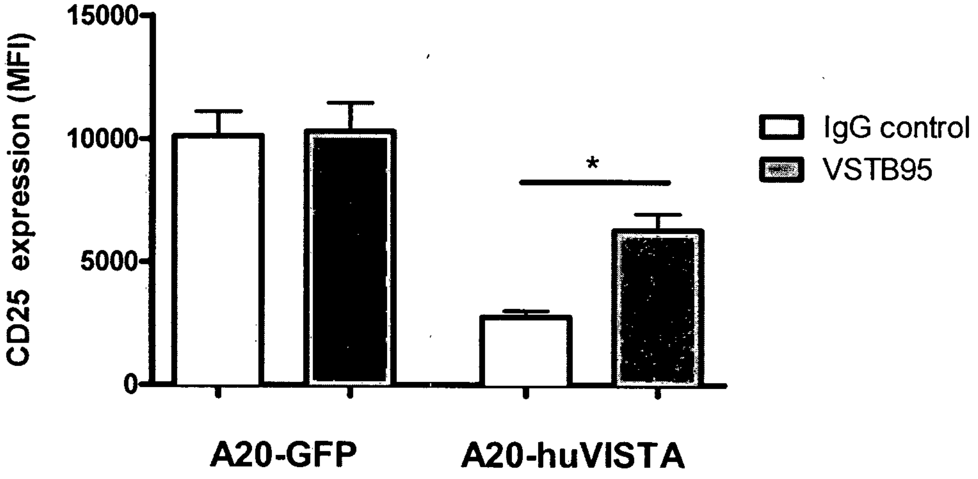

CD25 expression (MFI)
15000
10000
5000
0
A20-GFP
A20-huVISTA
*
IgG control
VSTB95

Figure 18

Figure 19A

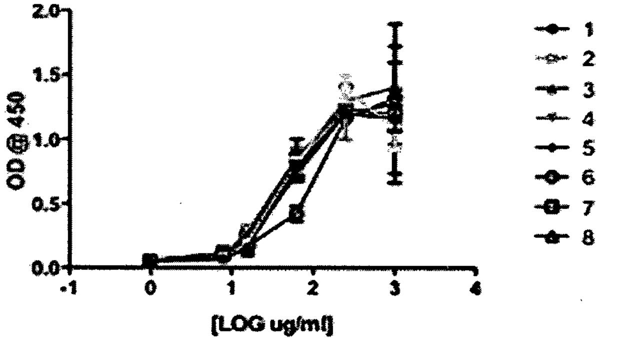


Figure 19B

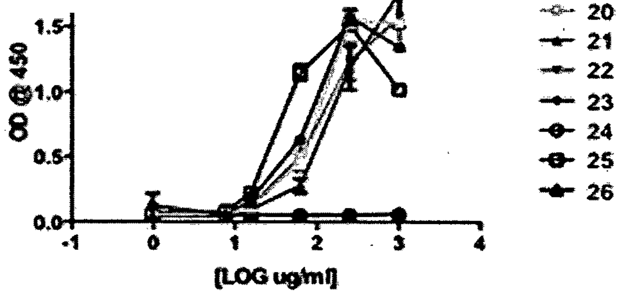


Figure 19C

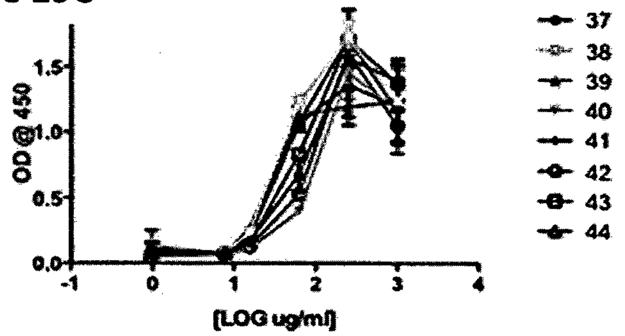


Figure 19D

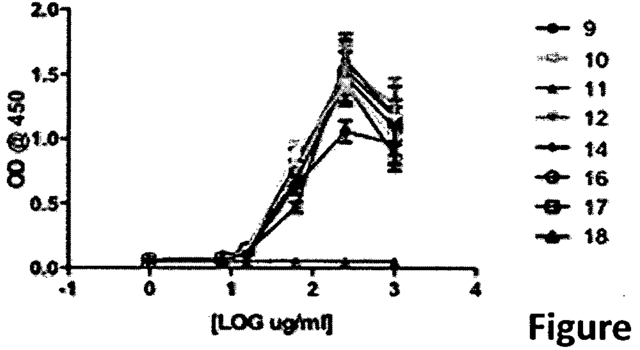


Figure 19E

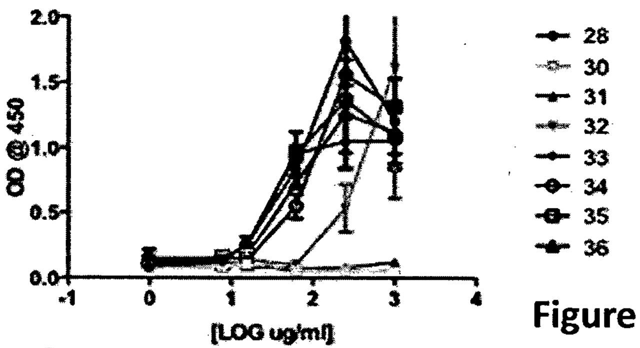


Figure 19F

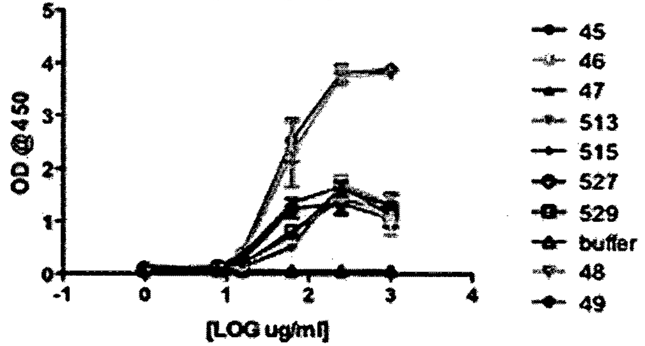


Figure 20A

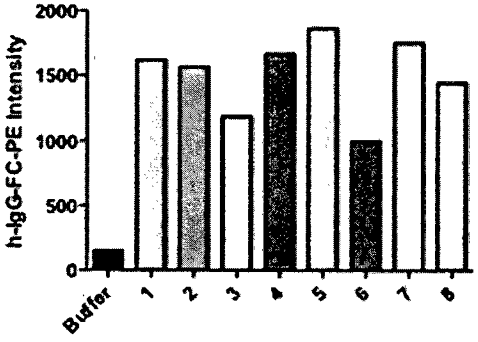


Figure 20D

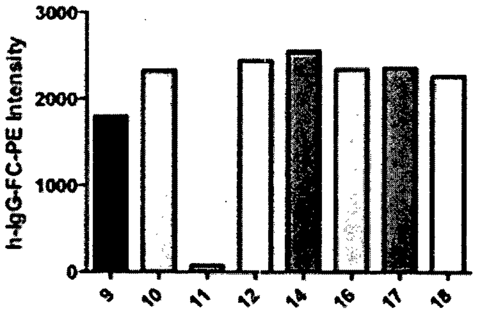


Figure 20B

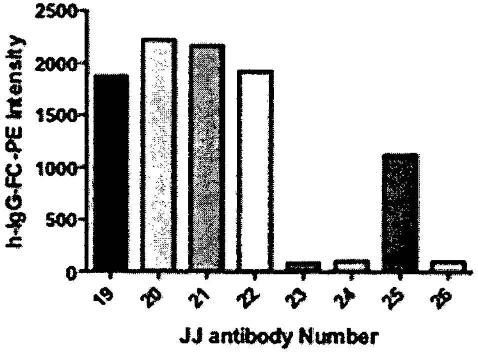


Figure 20E

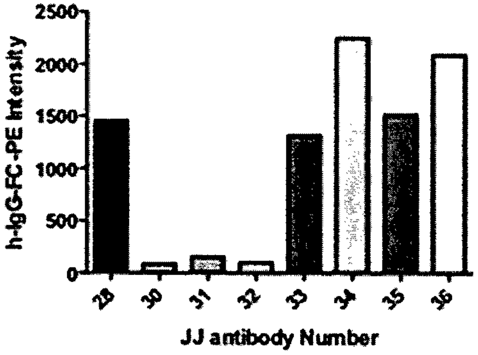


Figure 20C

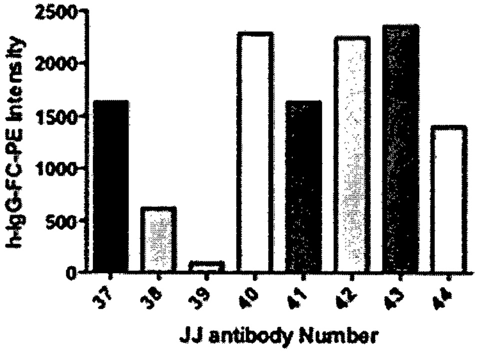


Figure 20F

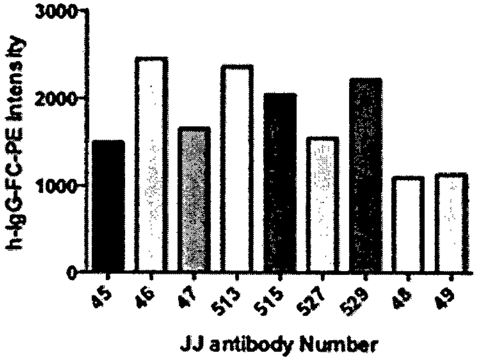


Figure 21A

Donor 60-> Donor 62

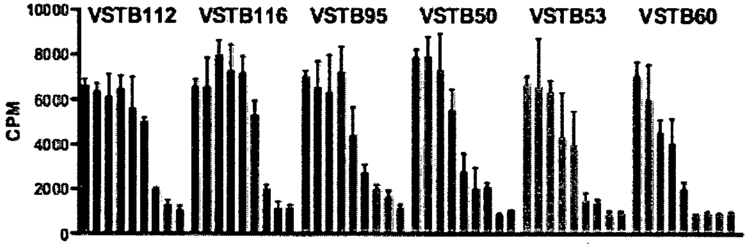
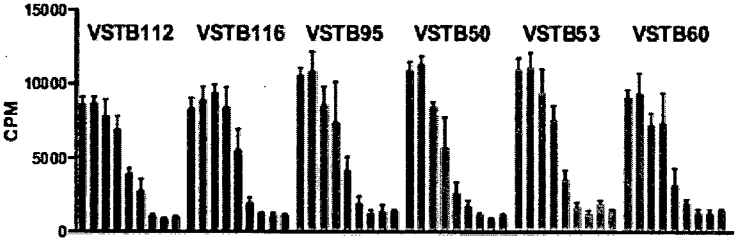


Figure 21B

Donor 62-> Donor 60



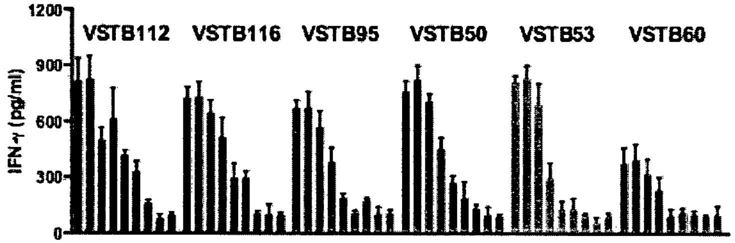


Figure 21C

30-10-3-1-0.3-0.1-0.03-0.01-0
µg/ml

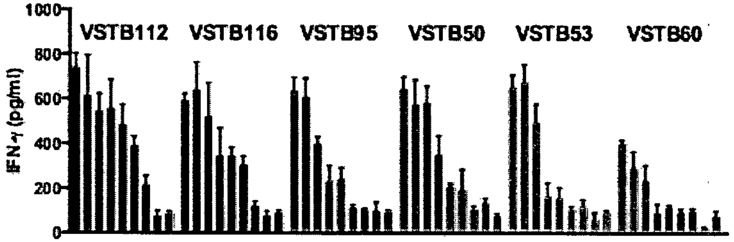


Figure 21D

Donor #1

Figure 22A

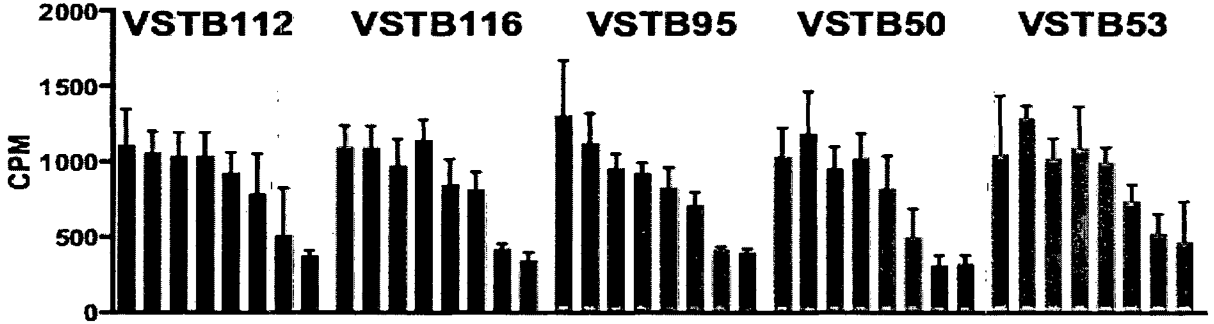
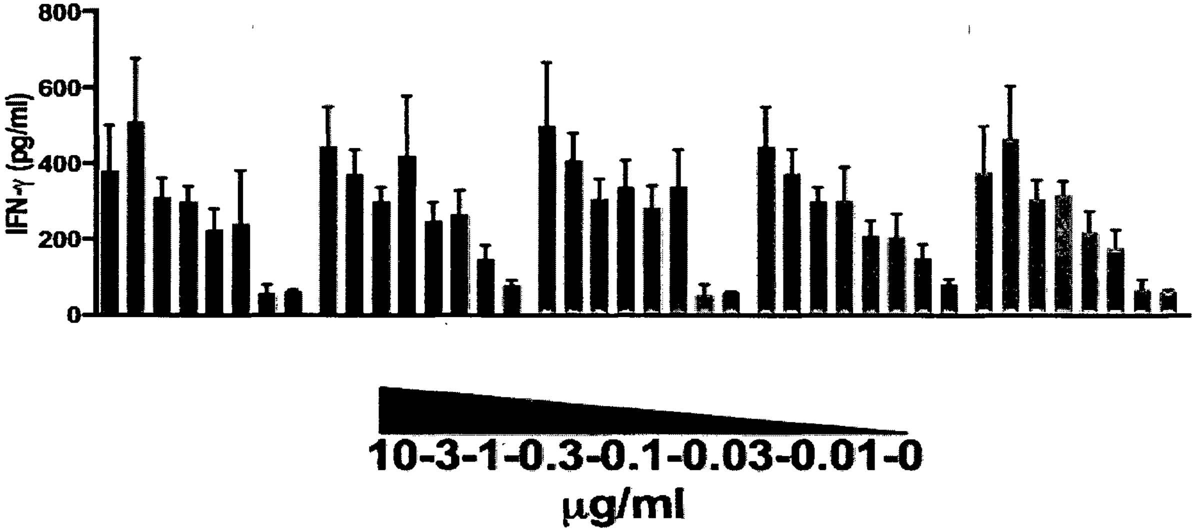


Figure 22B



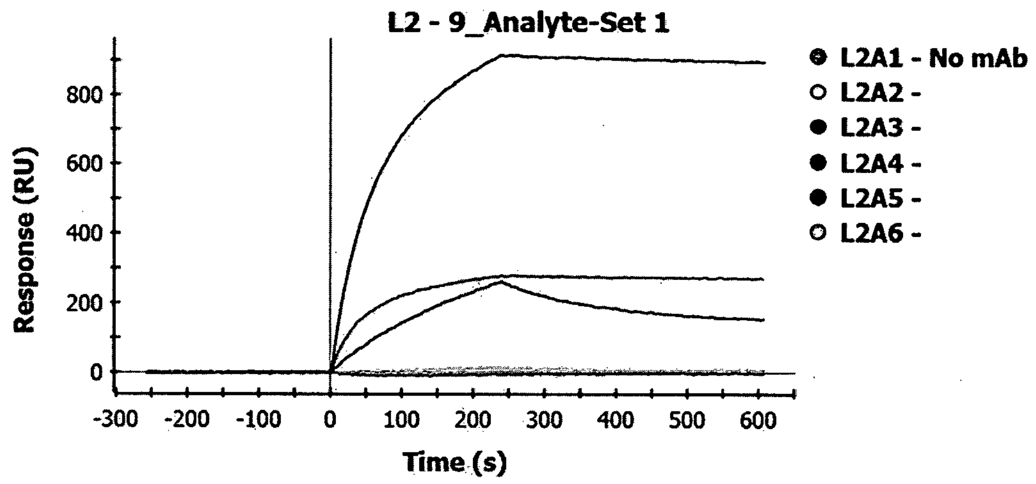

L2 - 9_Analyte-Set 1
Response (RU)
800
600
400
200
0
-300
-200
-100
0
100
200
300
400
500
600
Time (s)
⊕ L2A1 - No mAb
○ L2A2 -
● L2A3 -
● L2A4 -
● L2A5 -
○ L2A6 -

Figure 23

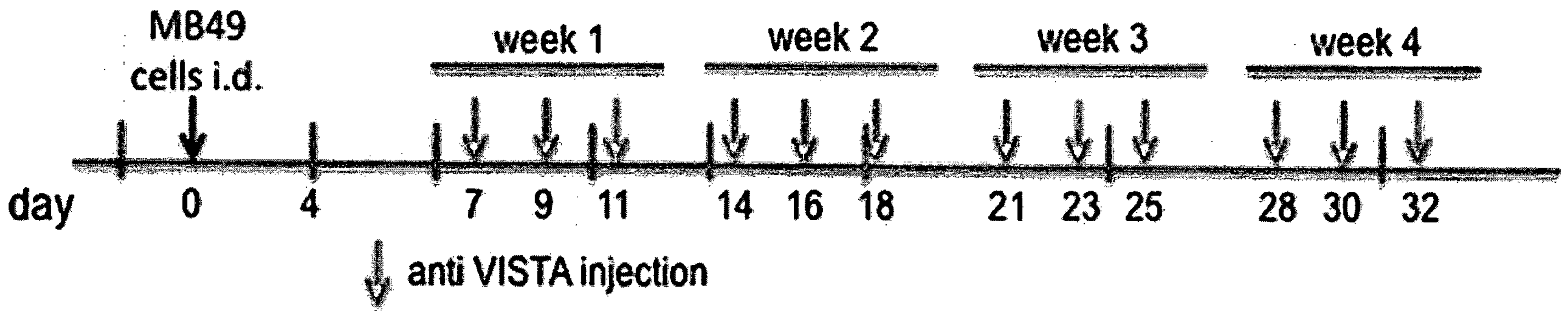

MB49
cells i.d.
week 1
week 2
week 3
week 4
day
0
4
7
9
11
14
16
18
21
23
25
28
30
32
anti VISTA injection

Figure 24

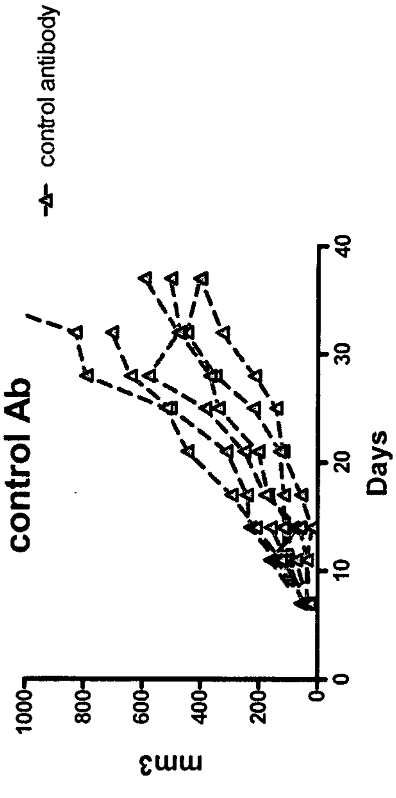


Figure 25A

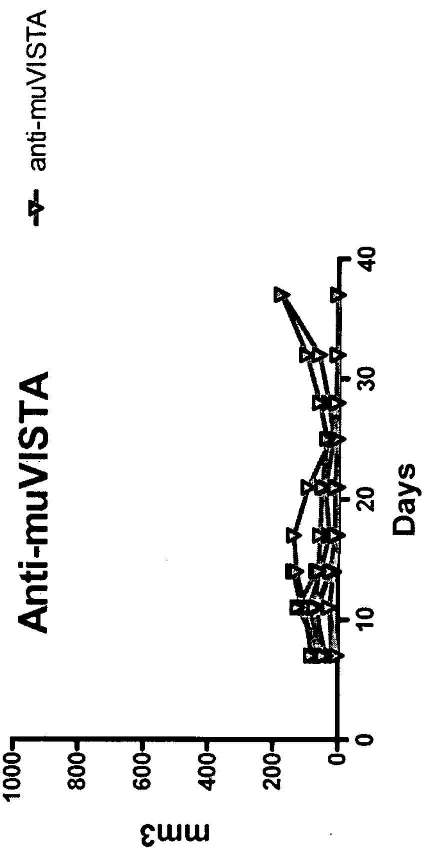


Figure 25B

MGVPTALEAGSWRWGSLLFALFLAASLGPVAAFKVATPYSLYVCP
EGQNVTLTCRLLGPVDKGHDVTFYKTWYRSSRGEVQTCSERRPI
RNLTFQDLHLHHGGHQAANTSHDLAQRHGLESASDHHGNFSIT
MRNLTLLDSGLYCCLVVEIRHHHSEHRVHGAMELQVQTGKDAPS
NCVVYPSSSQESENITAAALATGACIVGILCLPLILLLVYKQ
RQAASNRRAQELVRMDSNIQGIENPGFEASPPAQGIPEAKVRHP
LSYVAQRQPSESGRHLLSEPSTPLSPPGPGDVF (SEQ ID NO:46)

Figure 26

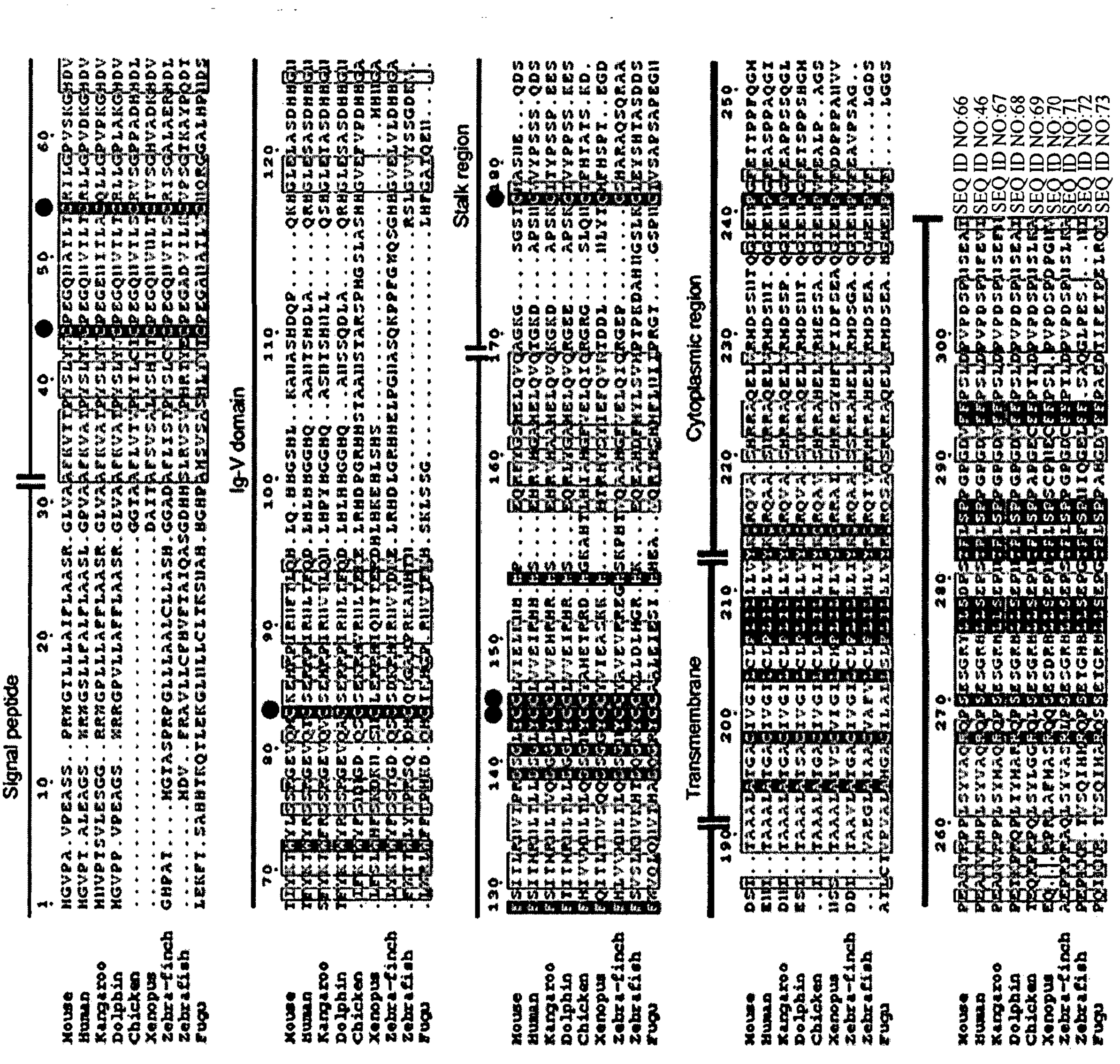

Signal peptide
Ig-V domain
Stalk region
Transmembrane
Cytoplasmic region
Mouse
Human
Kangaroo
Dolphin
Chicken
Xenopus
Zebra-finch
Zebrafish
Fugu
SEQ ID NO:66
SEQ ID NO:46
SEQ ID NO:67
SEQ ID NO:68
SEQ ID NO:69
SEQ ID NO:70
SEQ ID NO:71
SEQ ID NO:72
SEQ ID NO:73

Figure 27


1 FKVAT PYGLY VCPEG QDVTL TCRLL GPVDK GHDVT FYKTW YRSSR GEVQT CSERR PIRDL
61 TFQDL HLHHG GHQAA DTSHD LAQRH GLESA SDHHG NFSIT MRNLT LLDSG LYCCL VVEIR
121 HHHSE HRVHG AMELQ VQTGK DAPSN CVVYP SSSQE SRNIT AAHHH HHH
0.50m
2m
10m
60m
< -90%
< -70%
< -50%
< -30%
< -10%
< 10%
< 30%
< 50%
< 70%
< 90%
> 90%

Figure 28

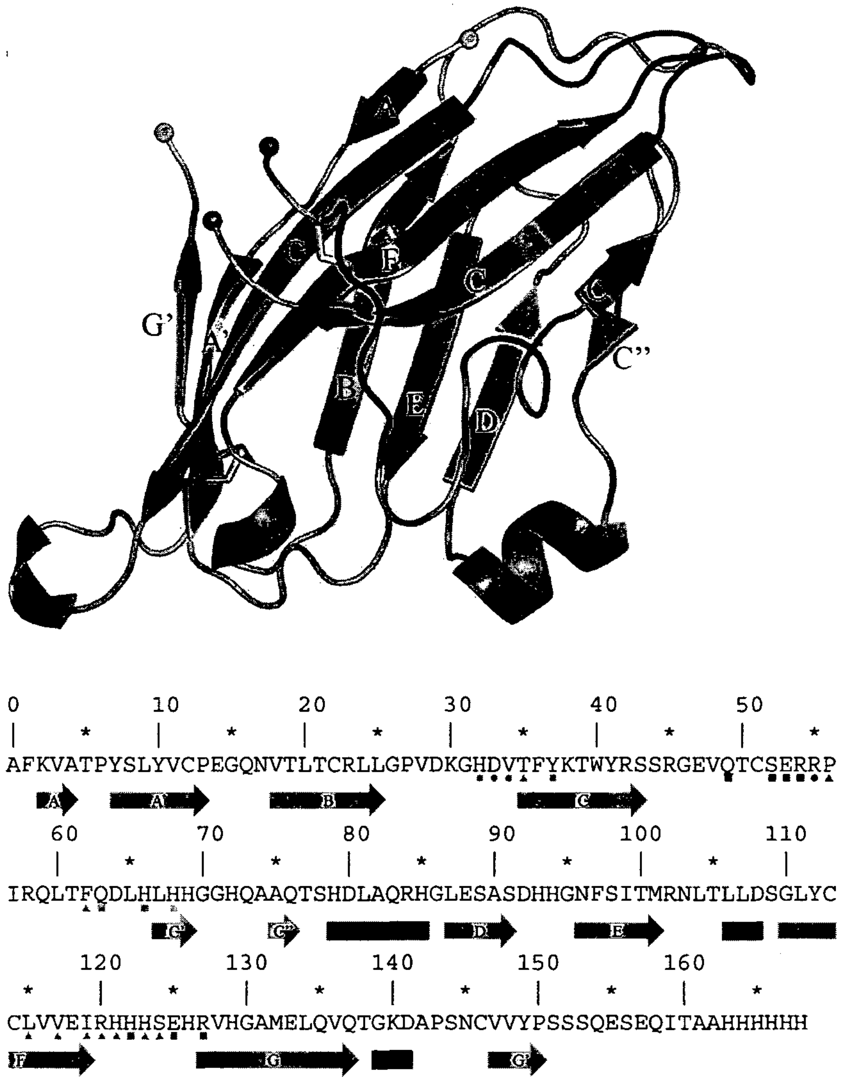
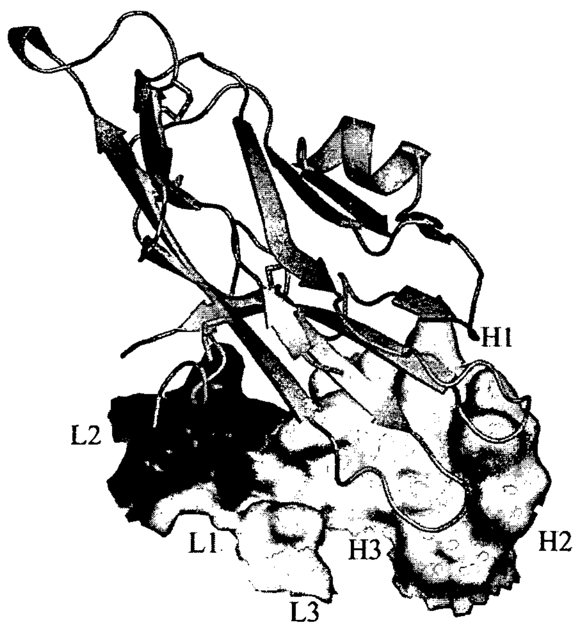

A
A'
B
C
C'
C''
D
E
F
G'
0 10 20 30 40 50
AFKVATPYSLYVCPEGQNVTLTCRLLGPVDKGHDVTFYKTWYRSSRGEVQTCSERRP
60 70 80 90 100 110
IRQLTFQDLHLHHGGHQAAQTSHDLAQRHGLESASDHHGNFSITMRNLTLLDSGLYC
120 130 140 150 160
CLVVEIRHHHSEHRVHGAMELQVQTGKDAPSNCVVYPSSSQESEQITAAHHHHHH
A'
A
B
C
G'
G''
D
E
F
G
G'

Figure 29



LC

```
          10        20        30        40        50        60
     *    |    *    |    *    |    *    |    *    |    *    |
DIQMTQSPSSLSASVGDRVTITCRASQSIDTRLNWYQQKPGKAPKLLIYSASSLQSGVPS
                              ■■                ■▲▲■■▲▲■

          70        80        90       100       110       120
     *    |    *    |    *    |    *    |    *    |    *    |
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYNPITFGQGTKVEIKRTVAAPSVFIFPP
                               ●
```

HC

```
          10        20        30        40        50        60
     *    |    *    |    *    |    *    |    *    |    *    |
QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGTANY
 ▲                         ▲ ▲■■                   ▲ ▲▲ ■ ■

          70        80        90       100       110
     *    |    *    |    *    |    *    |    *    |    *    |
AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSSYGWSYEFDYWGQGTLVTVSS
                                     ▲●▲■●▲▲ ■ ■■
```

Figure 30

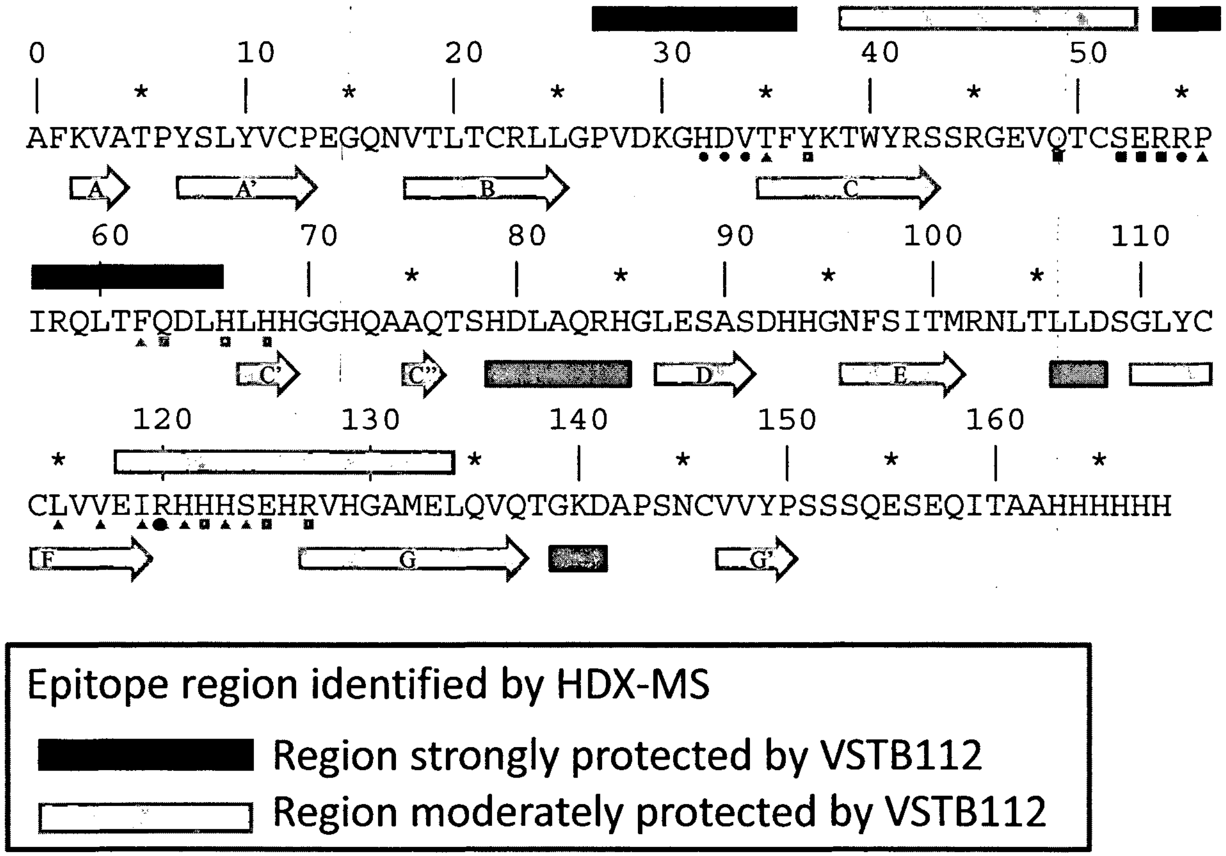
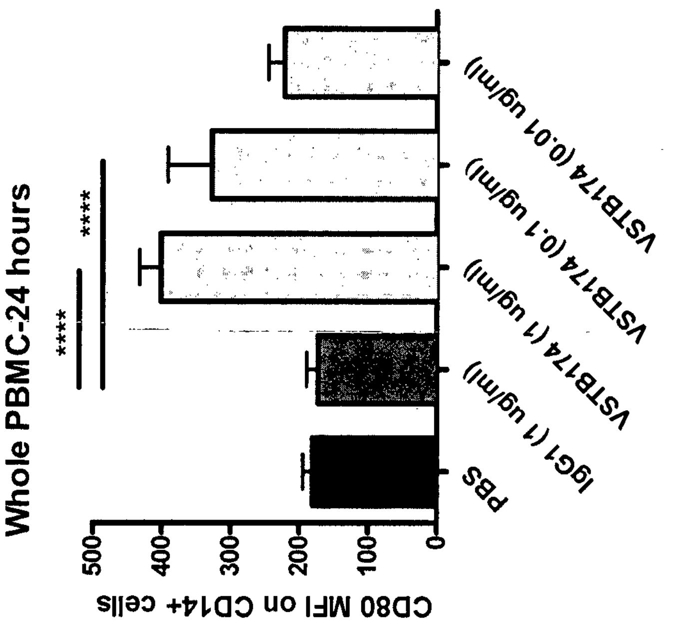

0
10
20
30
40
50
AFKVATPYSLYVCPEGQNVTLTCRLLGPVDKGHDVTFYKTWYRSSRGEVQTCSERRP
A
A'
B
C
60
70
80
90
100
110
IRQLTFQDLHLHHGGHQAAQTSHDLAQRHGLESASDHHGNFSITMRNLTLLDSGLYC
C'
C''
D
E
120
130
140
150
160
CLVVEIRHHHSEHRVHGAMELQVQTGKDAPSNCVVYPSSSQESEQITAAHHHHHH
F
G
G'
Epitope region identified by HDX-MS
Region strongly protected by VSTB112
Region moderately protected by VSTB112

Figure 31


Whole PBMC-24 hours
CD80 MFI on CD14+ cells
500
400
300
200
100
0
****
****
PBS
IgG1 (1 ug/ml)
VSTB174 (1 ug/ml)
VSTB174 (0.1 ug/ml)
VSTB174 (0.01 ug/ml)

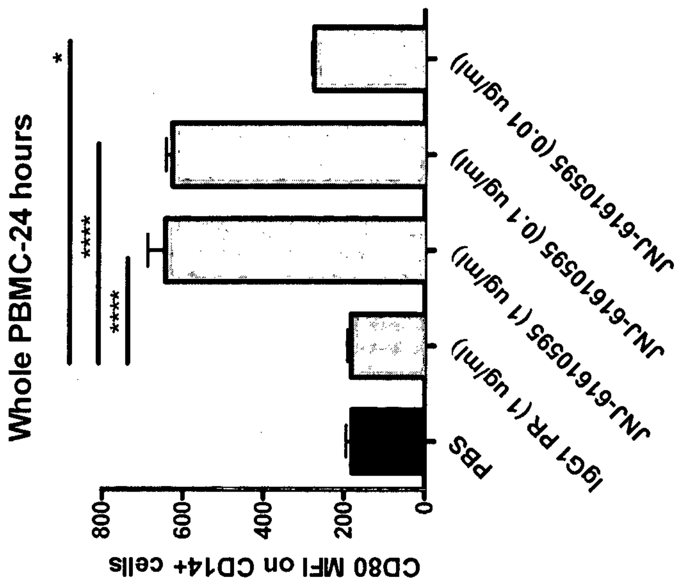

Whole PBMC-24 hours
CD80 MFI on CD14+ cells
800
600
400
200
0
*
****
****
PBS
IgG1 PR (1 ug/ml)
JNJ-61610595 (1 ug/ml)
JNJ-61610595 (0.1 ug/ml)
JNJ-61610595 (0.01 ug/ml)

Figure 32

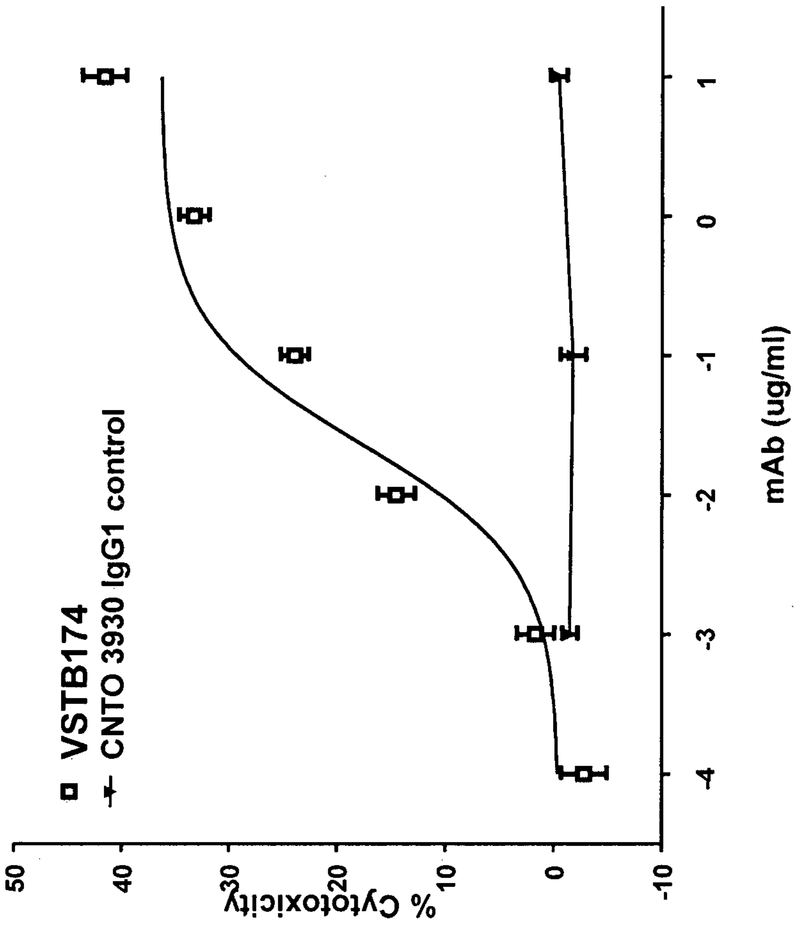

VSTB174
CNTO 3930 IgG1 control
% Cytotoxicity
50
40
30
20
10
0
-10
-4
-3
-2
-1
0
1
mAb (ug/ml)

Figure 33

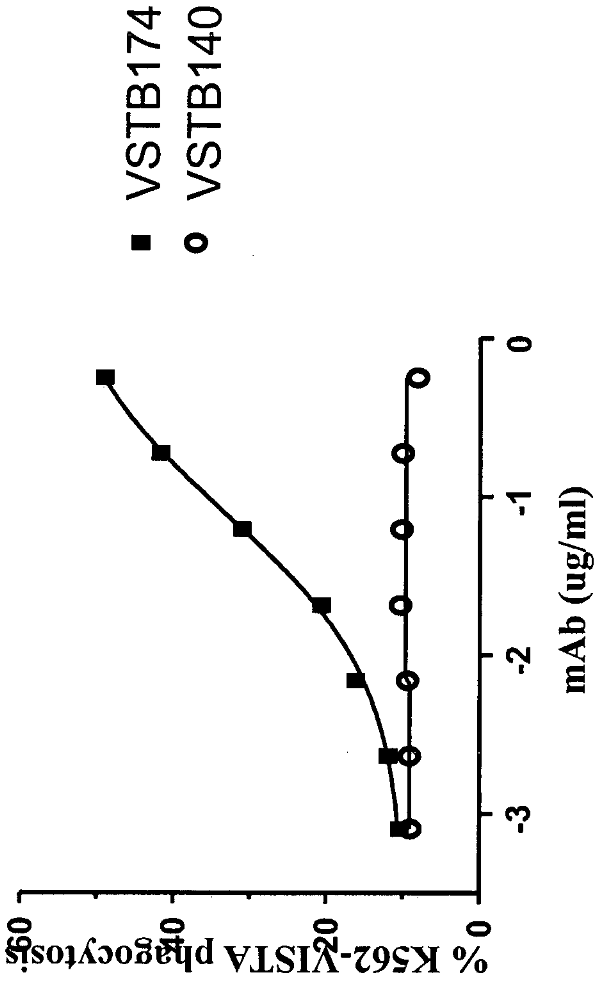

% K562-VISTA phagocytosis
0
20
40
60
-3
-2
-1
0
mAb (ug/ml)
■ VSTB174
o VSTB140

Figure 34

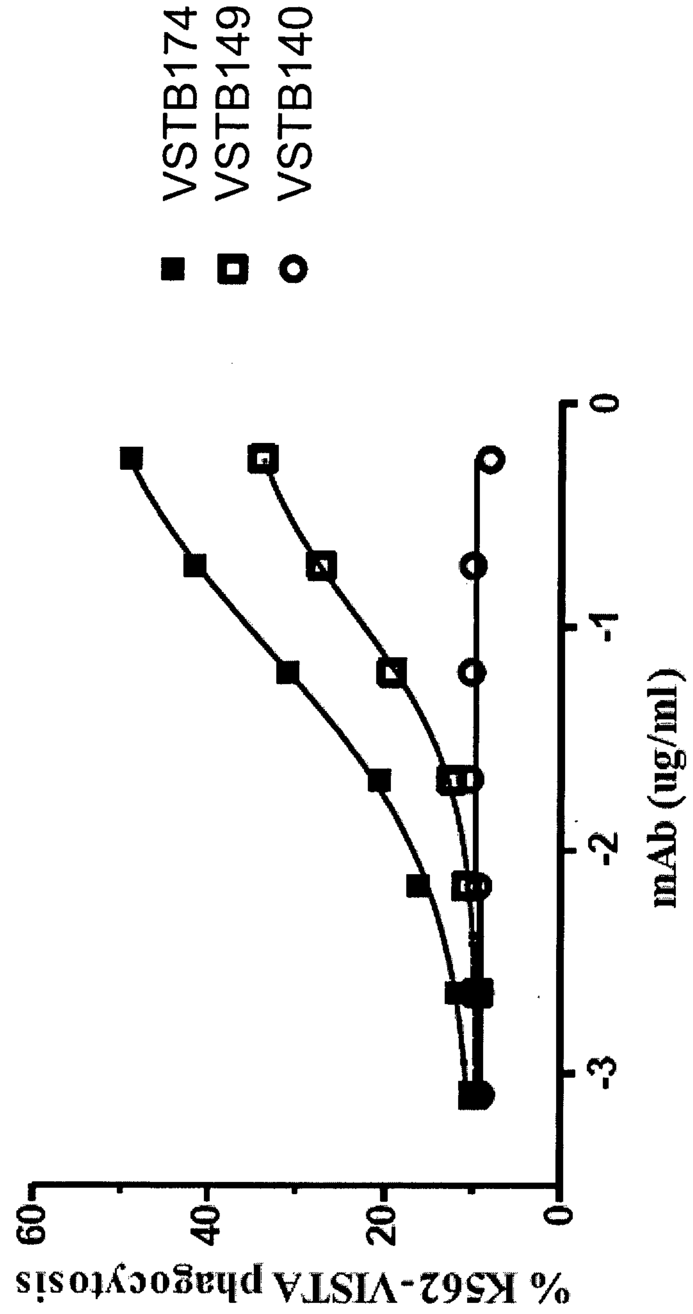

VSTB174
VSTB149
VSTB140
% K562-VISTA phagocytosis
60
40
20
0
-3
-2
-1
0
mAb (ug/ml)

Figure 35

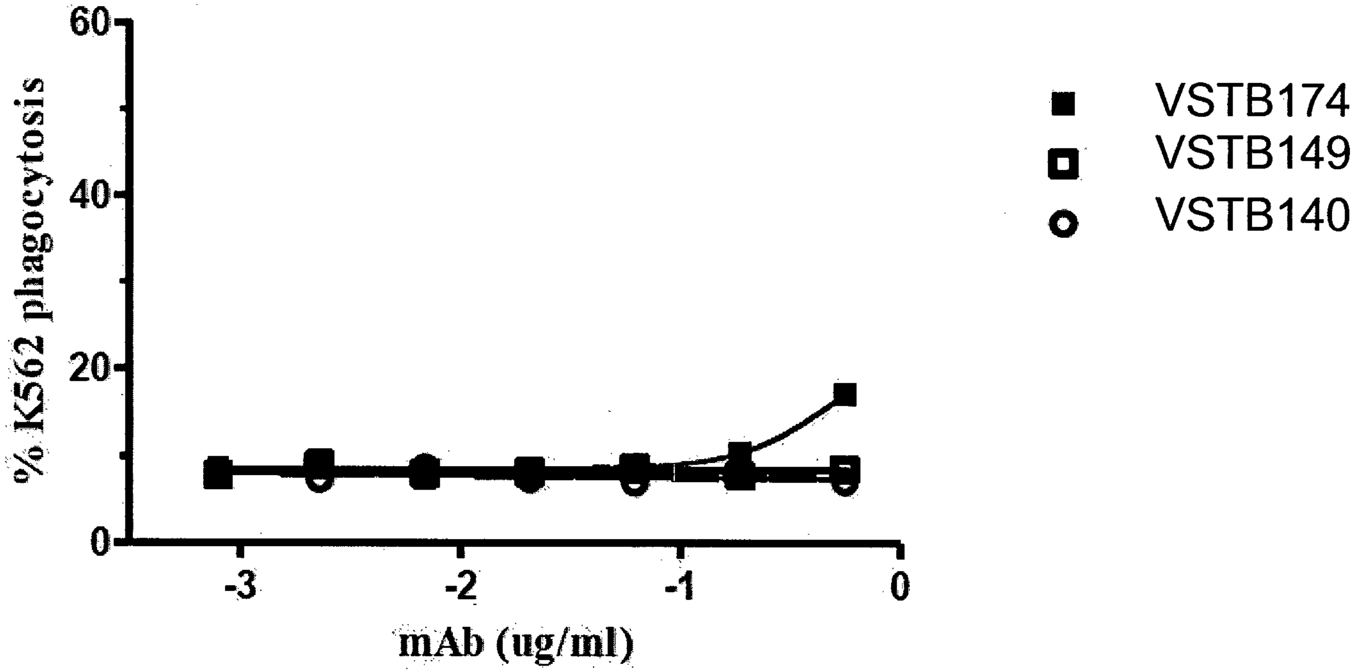

% K562 phagocytosis
60
40
20
0
-3
-2
-1
0
mAb (ug/ml)
■ VSTB174
□ VSTB149
○ VSTB140

Figure 36

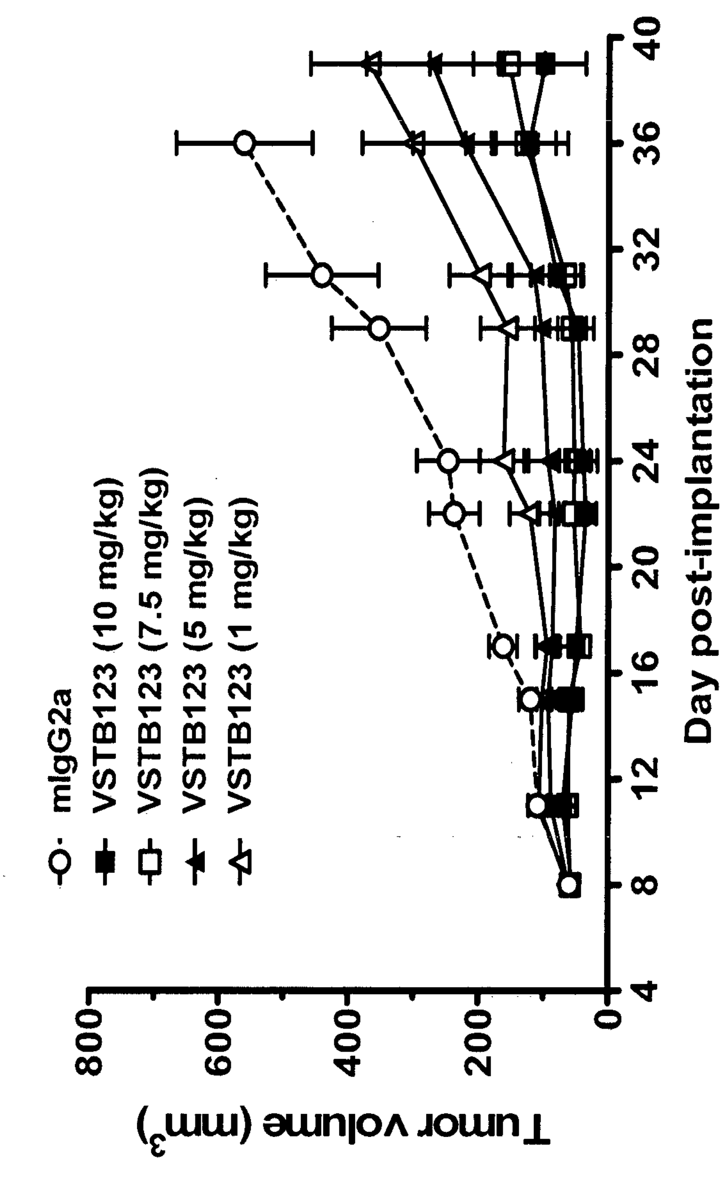

mIgG2a
VSTB123 (10 mg/kg)
VSTB123 (7.5 mg/kg)
VSTB123 (5 mg/kg)
VSTB123 (1 mg/kg)
Tumor volume (mm^3)
800
600
400
200
0
4
8
12
16
20
24
28
32
36
40
Day post-implantation

Figure 37

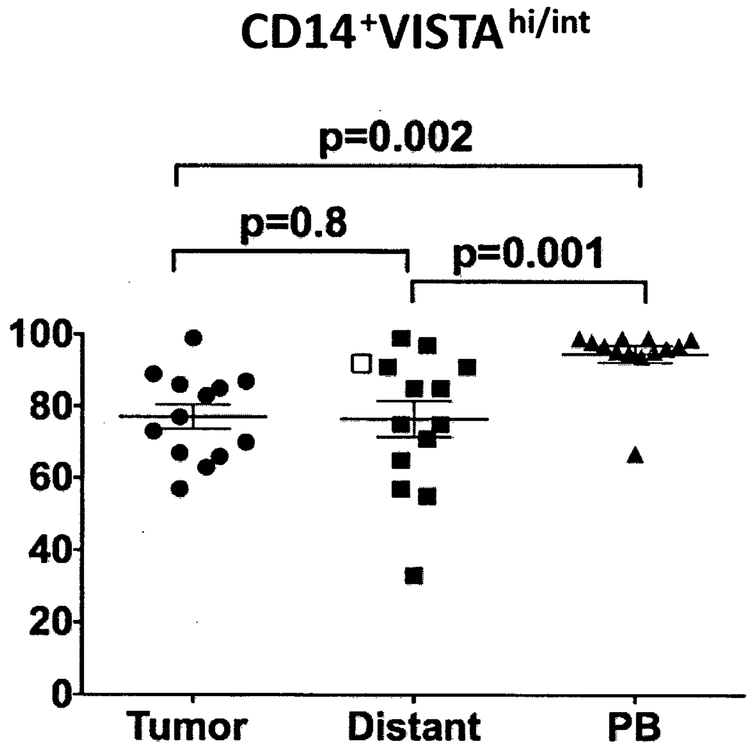

CD14+VISTA hi/int
p=0.002
p=0.8
p=0.001
100
80
60
40
20
0
Tumor
Distant
PB

Figure 38

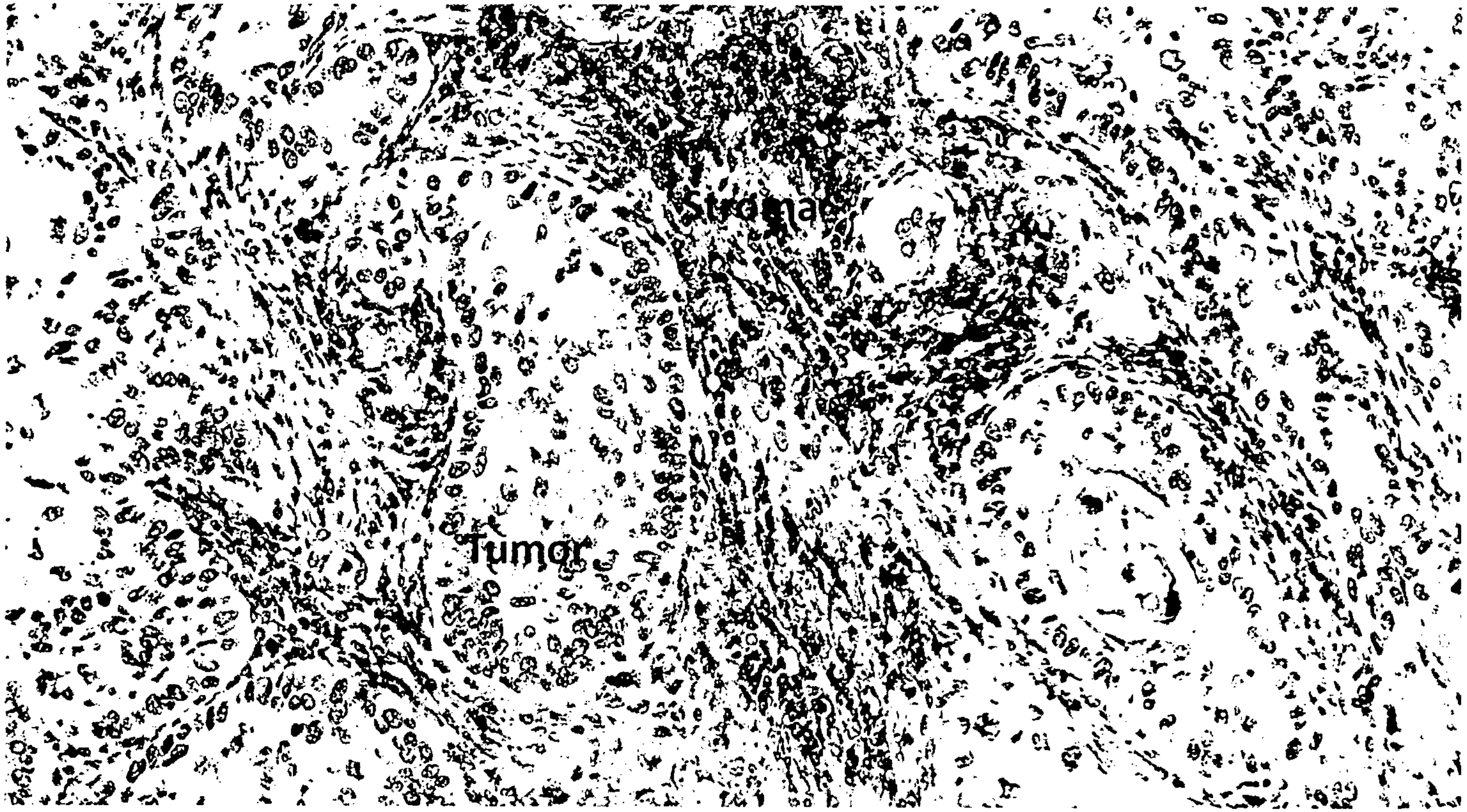


Figure 39

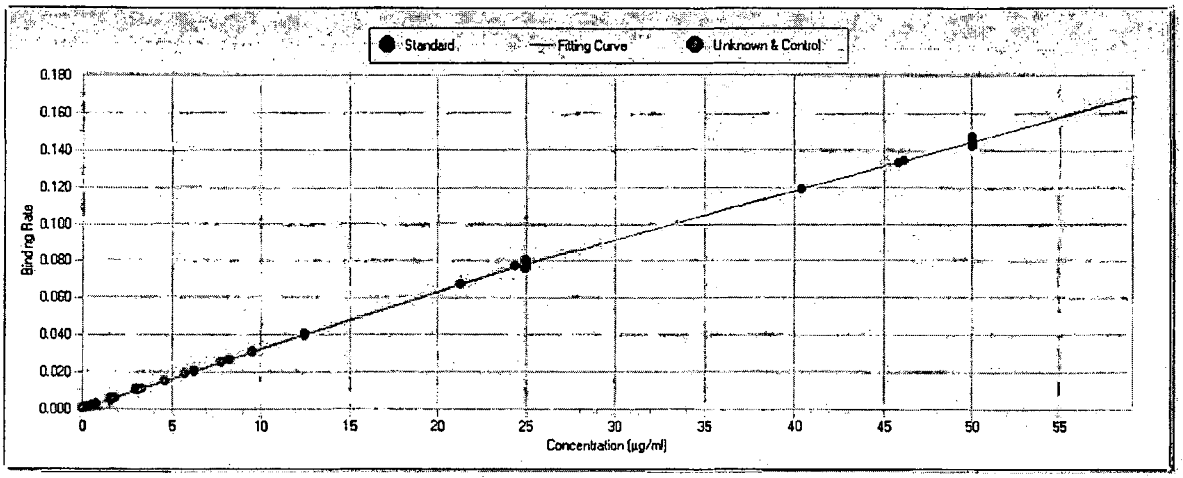
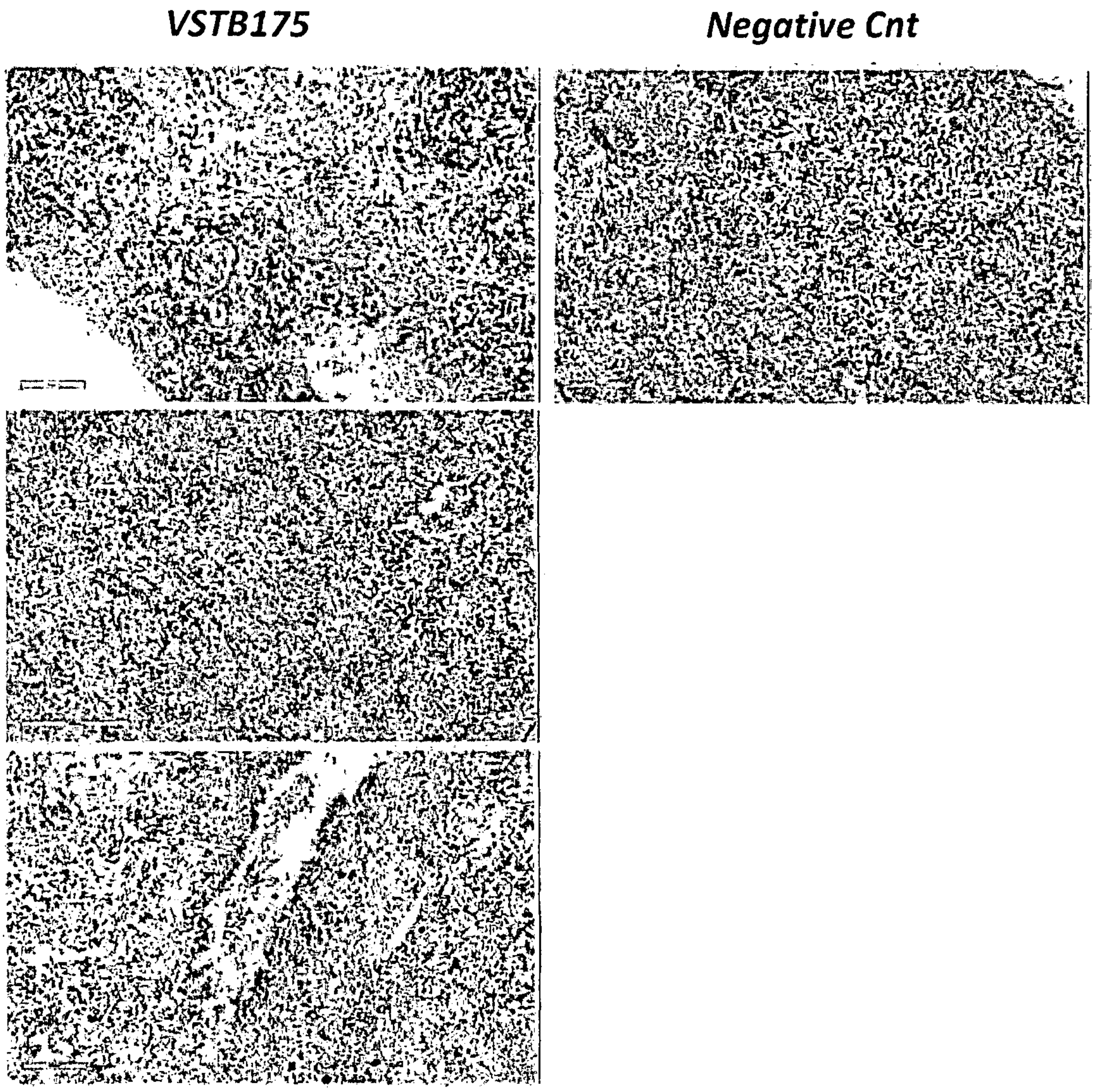

● Standard
Fitting Curve
● Unknown & Control
Binding Rate
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0
5
10
15
20
25
30
35
40
45
50
55
Concentration (µg/ml)

FIG. 40



VSTB175

Negative Cnt

FIG. 41

FIG. 42

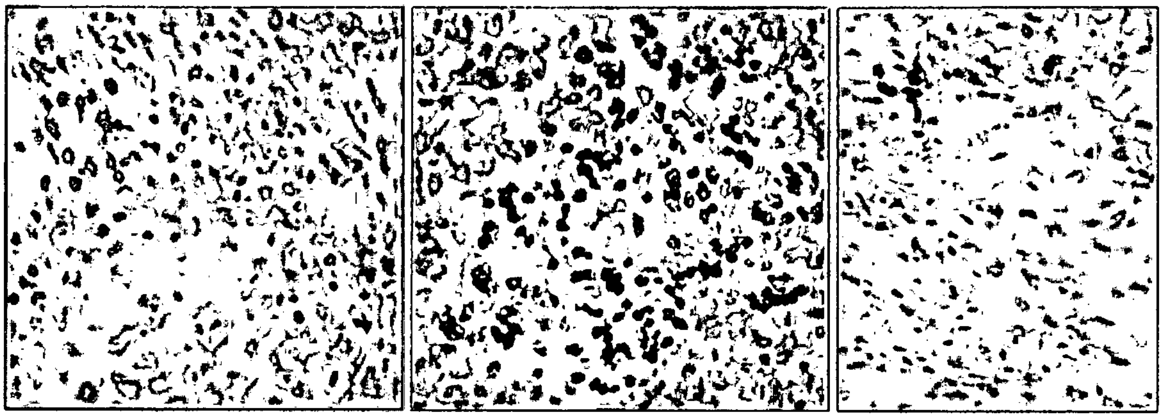


FIG. 43

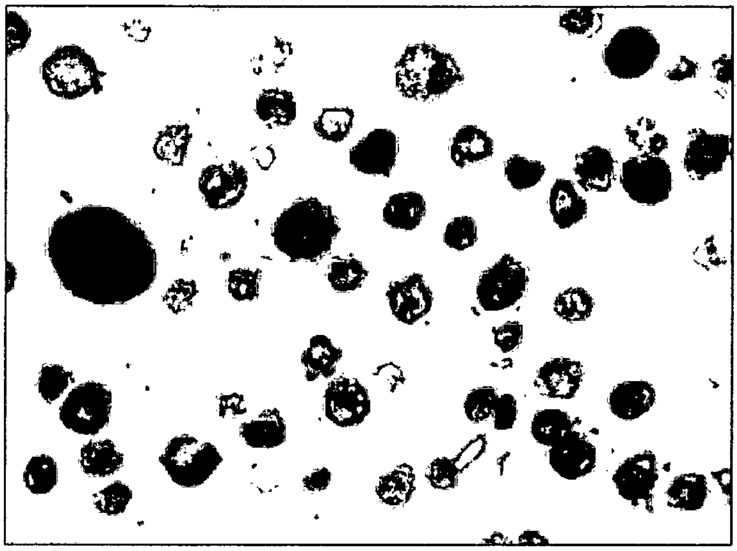


FIG. 44

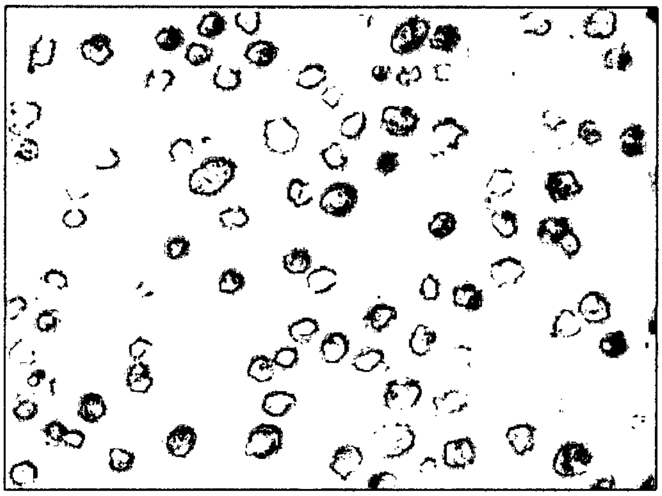


FIG. 45

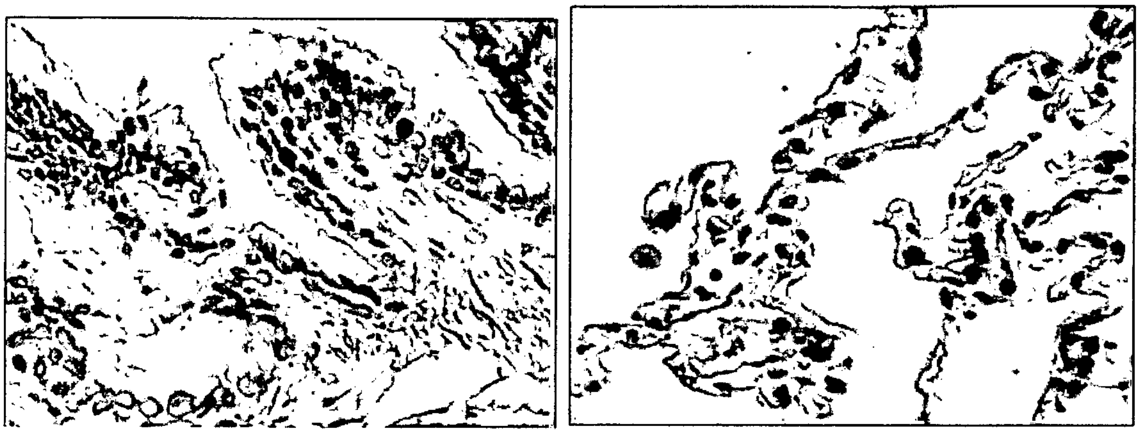


FIG. 46

FIG. 47

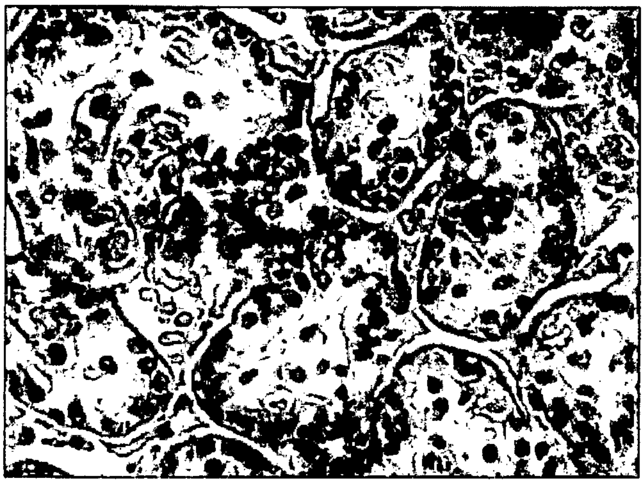


FIG. 48

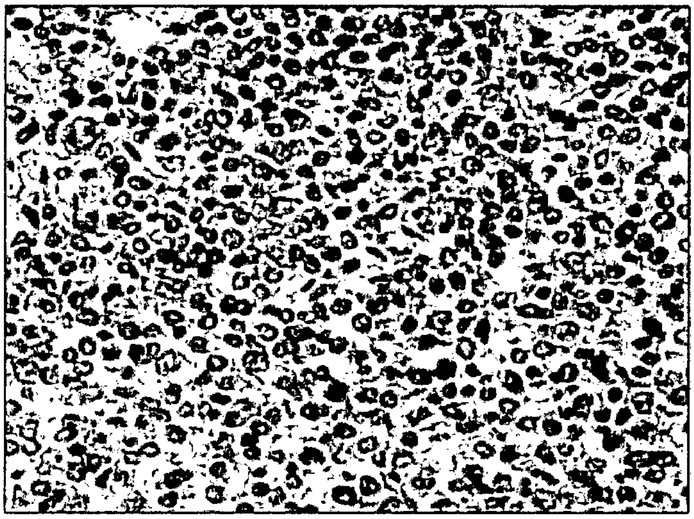


FIG. 49

FIG. 50

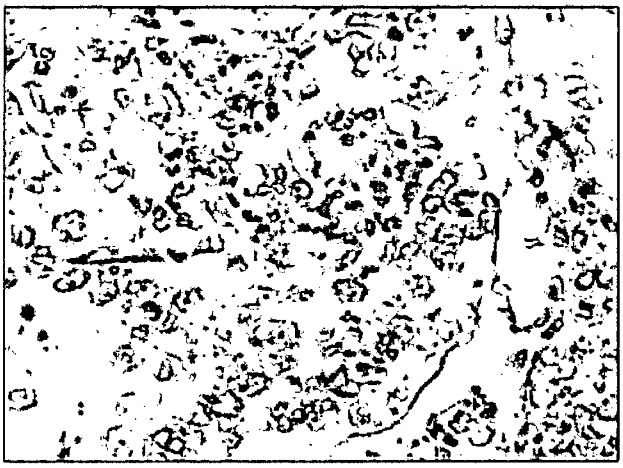


FIG. 51

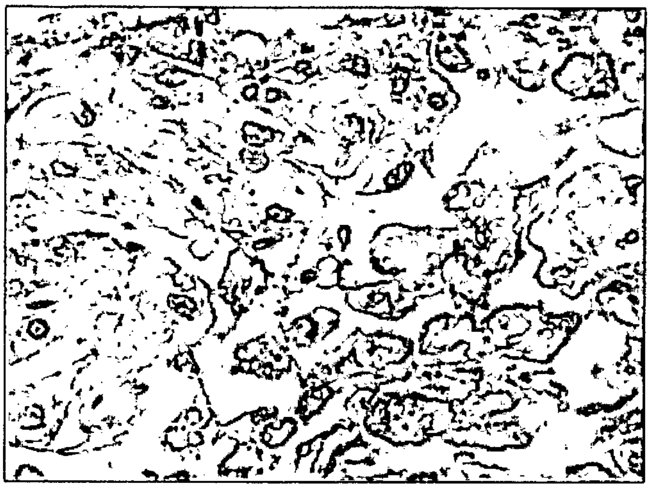


FIG. 52

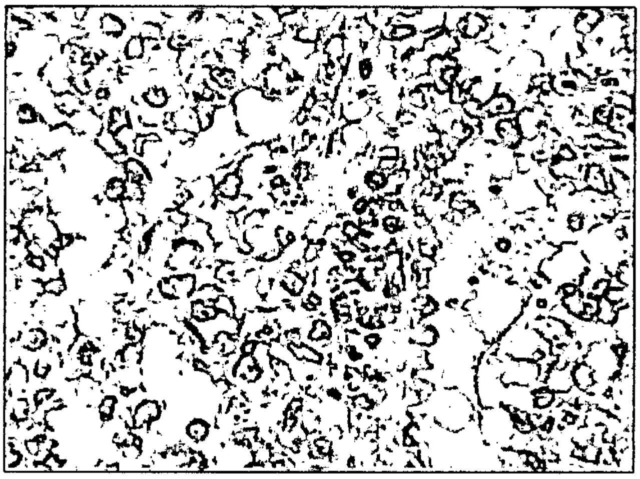


FIG. 53