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(54) **Titre : ANTICORPS ANTI-MUC16 ET LEURS PROCEDES D'UTILISATION**
(54) **Title: ANTIBODIES TO MUC16 AND METHODS OF USE THEREOF**

Peptide 1 near Cleavage Site:
NFSPLARRVDRVAIYEE (SEQ ID NO:01)

Peptide 2 before Transmembrane:
TLDRSSVLVDGYSPNRNE (SEQ ID NO:02)

Peptide 3 inside Transmembrane:
CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03)

(57) **Abrégé/Abstract:**
The invention provides antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

ABSTRACT

The invention provides antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

ANTIBODIES TO MUC16 AND METHODS OF USE THEREOF

This invention was made with United States government support under PO1-CA52477-16 awarded by the United States Public Health Service (US PHS). The United States government has certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

BACKGROUND OF THE INVENTION

Cell surface markers and shed antigens are used in the diagnosis of several cancers. For example, the CA125 antigen, recognized by the OC125 antibody, is a tissue-specific, circulating antigen expressed in ovarian cancer. The CA125 antigen is encoded by the MUC16 gene, cloned by Lloyd and Yin. The full-length gene describes a complex tethered mucin protein present primarily in a variety of gynecologic tissues, especially neoplasms. OC125 and other related antibodies react with glycosylation-dependent antigens present exclusively in the cleaved portion of the molecule.

A serum assay can detect elevated levels of the circulating CA125 antigen in many epithelial ovarian cancer patients, and this antigen, derived using the ovarian cell line OVCA433, is recognized by the OC125 antibody (1-2). The detection of circulating CA125 in serum has proven to be a useful tool for the management of ovarian cancer patients and clinical trials (3-4). However, CA125 is neither sufficiently sensitive nor specific for general cancer screening (5-6). A variety of CA125 linked antibodies including VK8 and M11 have subsequently been defined as present on ovarian cancer cells (7-9). Although these antibodies have been used to develop serum assays and various other studies in ovarian cancer, they have significant shortcomings for clinical use in screening or tissue delivery. These antibodies are not useful as screening tools, nor can they

detect the proximal residual MUC16 protein fragment after cleavage. This has limited their diagnostic and therapeutic applications.

For example, OC125, M11, and most other antibodies prepared against ovarian cancer cell extracts are directed at complex, glycosylation-dependent antigens. These antigens are exclusively present in the shed portion of MUC16 and cannot be employed to follow the biology of the proximal portion of MUC16 and may not accurately reflect tissue distribution since the glycosylation patterns can vary substantially among tissues. Because the vast majority of MUC16-reactive antibodies, including OC125, react with the glycosylation-dependent antigen present exclusively in the cleaved portion of the molecule, the true distribution of MUC16 expression is not known (21). There is currently no antibody available to track the fate of the remaining MUC16 protein fragment after cleavage and CA125 release.

Thus, there remains a need for the identification of antibodies that are directed against sequences in the peptide backbone of MUC16, and that are useful for diagnosis and treatment of cancers in which MUC16 is expressed and/or overexpressed.

SUMMARY OF THE INVENTION

The invention provides an antibody, or an antigen-binding fragment thereof, that specifically binds to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from the group of a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19). In one embodiment, the antibody internalizes into a cell. While not intending to limit the invention to a particular sequence of MUC 16 ectodomain, in one embodiment, the MUC16 ectodomain polypeptide comprises a polypeptide selected from the group of Polypeptide 1 NFSPLARRVDRVAIYEE (SEQ ID NO:01) and Polypeptide 2 TLDRSSVLVDGYSPNRNE (SEQ ID NO:02). In another embodiment, the antibody lacks specific binding to a glycosylated MUC16 extracellular domain. In yet a further embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:06, and a variable light (V_L) chain encoded by SEQ ID NO:07. In yet another alternative embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:04, and a variable light (V_L) chain encoded by SEQ ID NO:05. In a further embodiment, the antibody specifically binds to the Polypeptide 1 (SEQ ID NO:01) of the MUC16 ectodomain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain

encoded by SEQ ID NO:08, and a variable light (V_L) chain encoded by at least one of SEQ ID NO:09 and SEQ ID NO:10. In one embodiment, the MUC16 cytoplasmic domain polypeptide comprises VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18). More preferably, but without limitation, the MUC16 cytoplasmic domain polypeptide comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03). In an alternative embodiment, the MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide comprises CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19). More preferably, but without limitation, the MUC16 extracellular domain polypeptide comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15). In yet another alternative embodiment, the antibody specifically binds to the Polypeptide 4 (SEQ ID NO:15) of the MUC16 extracellular domain polypeptide, and wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:11, and a variable light (V_L) chain encoded by SEQ ID NO:12. In a further alternative embodiment, the antibody is selected from the group of a chimeric antibody, a monoclonal antibody, a recombinant antibody, an antigen-binding fragment of a recombinant antibody, a humanized antibody, and an antibody displayed upon the surface of a phage. In another embodiment, the antigen-binding fragment is selected from the group of a Fab fragment, a F(ab')2 fragment, and a Fv fragment. In an alternative embodiment, the antibody, or antigen-binding fragment thereof, is covalently linked to a cytotoxic agent or a prodrug of a cytotoxic agent. In a preferred embodiment, the antibody is a monoclonal antibody produced by a hybridoma cell line.

The invention also provides an isolated monoclonal antibody, or an antigen-binding fragment thereof, produced by a hybridoma cell line, wherein the antibody specifically binds to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from the group of a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19). In one embodiment, the MUC16 ectodomain polypeptide comprises Polypeptide 1 (SEQ ID NO:01) and the antibody is selected from the group of 9B11.20.16, 10A2, 2F4, 23D3, 30B1, and 31B2. In an alternative embodiment, the MUC16 ectodomain polypeptide comprises Polypeptide 2 (SEQ ID NO:02), and wherein the antibody is selected from the group of 4H11.2.5 , 13H1, 29G9, 9C9.21.5.13, 28F8, 23G12, 9C7.6, 11B6, 25G4, 5C2.17, 4C7, 26B2, 4A5.37, 4A2, 25H3, and 28F7.18.10. In yet a further embodiment, the MUC16 cytoplasmic domain polypeptide comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03), and wherein the antibody is selected from the group of 31A3.5.1, 19D1, 10F6, 22E10, 22F1, 3H8, 22F11, 4D7, 24G12, 19G4, 9A5, 4C2, 31C8, 27G4, and 6H2. In another alternative embodiment, the MUC16 extracellular domain

polypeptide comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15), and wherein the antibody is selected from the group of 24B3 and 9C7.

The invention additionally provides a composition comprising (a) any one or more of the antibodies, or antigen-binding fragments thereof, that are described herein, and (b) a pharmaceutically acceptable carrier.

Also provided by the invention is a hybridoma cell line that produces a monoclonal antibody that specifically binds to a polypeptide, or antigenic portion thereof, selected from the group of a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19).

The invention additionally provides a method for detecting a disease that comprises overexpression of MUC16 in a subject, comprising a) providing i) a sample from a subject, and ii) any one or more of the antibodies, or antigen-binding fragments thereof, that are described herein, b) contacting the sample with the antibody under conditions for specific binding of the antibody with its antigen, and c) detecting an increased level of binding of the antibody to the sample compared to a control sample lacking the disease, thereby detecting the disease in the subject. In one embodiment, the disease is cancer. In a preferred embodiment, the cancer is selected from the group of ovarian cancer and breast cancer. While not intending to limit the method of detection, in one embodiment, detecting binding of the antibody to the sample is immunohistochemical, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, immunoprecipitation, and/or radiographic imaging.

Also provided herein is a method for treating a disease that comprises overexpression of MUC16, comprising administering to a subject having the disease a therapeutically effective amount of any one or more of the antibodies, or antigen-binding fragments thereof, that are described herein. In one embodiment, the disease is cancer, as exemplified by ovarian cancer and breast cancer.

The invention also provides an isolated antibody, or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSLCNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD. In one embodiment, the antibody is selected from the group of a monoclonal antibody, a chimeric antibody, a recombinant antibody, an antigen-binding fragment of a recombinant antibody, a humanized antibody, and an

antibody displayed upon the surface of a phage. In a preferred embodiment, the antibody is a monoclonal antibody produced by hybridoma cells selected from the group of 12B10-3G10, 10C4-3H5, 10C4-1F2, 10C4-2H8, 10C4-1G7, 17F2-3G5, 17F2-3F6, 17F2-2F9, 17F2-1E11, 12B10-3F7, 12B10-2F6, 12B10-2F10, 25E9-3, 25E9-5, 25E9-1, 25E9-16, 21B8-1H11, 21B8-3G6, 21B8-3H9, 21B8-1G8, 21E1-1E3, 21E1-1G9, 21E1-2G7, 21E1-3G12, 4H1-2E1, 4H1-2E3, 4H1-3E1, 4H1-3H3, 15A8-2E2, 15A8-2E10, 15A8-2E11, 15A8-3D2, 22B5-1F6, 22B5-3G9, 22B5-2G8, and 22B5-3F11. In a particular embodiment, the MUC16 polypeptide is TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), and the antibody comprises a variable heavy (V_H) chain sequence SEQ ID NO:27, and a variable light (V_L) chain sequence SEQ ID NO:29, such as the monoclonal antibody produced by hybridoma cell 12B10-3G10. In an alternative embodiment, the antigen-binding fragment is selected from the group of a Fab fragment, a F(ab')2 fragment, and a Fv fragment. In a more preferred embodiment, the antibody, or antigen-binding fragment thereof, is covalently linked to a cytotoxic agent and/or to a prodrug of a cytotoxic agent. In a further embodiment, the antibody specifically binds to human MUC16 (SEQ ID NO:25). In another embodiment, the antibody internalizes into a cell. In an alternative embodiment, the antibody lacks specific binding to a glycosylated MUC16 extracellular domain.

The invention also provides a composition comprising (a) any one or more of the invention's antibodies and/or antigen-binding fragments thereof, and (b) a pharmaceutically acceptable carrier.

The invention further provides a hybridoma cell that produces an antibody, or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSLCNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD.

The invention also provides an isolated nucleotide sequence comprising a polynucleotide that encodes at least one of a variable heavy (V_H) chain sequence and the variable light (V_L) chain sequence of an antibody that specifically binds to a MUC16 polypeptide, wherein the MUC16 polypeptide is selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSLCNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD. In one embodiment, the MUC16 polypeptide is TLDRKSVFVDGYSQNRDD (SEQ ID NO:21) and the polynucleotide

encoding the variable heavy (V_H) chain sequence comprises SEQ ID NO:26, and wherein the polynucleotide encoding the variable light (V_L) chain sequence comprises SEQ ID NO:28.

The invention also provides a method for producing an antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, comprising administering to a subject an immunologically effective amount of a MUC16 polypeptide selected from the group of a) TLDRKSVFVDGYSQNRDD (SEQ ID NO:21), b) KSYFSDCQVLAFRSVSNNNNHTGVDSLCNFSPL (SEQ ID NO:22), c) SLYSNCRLASLRPKKNGTATGVNAICSYHQN (SEQ ID NO:23), d) KSYFSDCQVNNFRS, e) TLDRSSVLVDGYSQNRDD, and f) TLDRSSVLVDGYSQNRDD.

The invention additionally provides a method for identifying a subject as having disease, comprising determining the level, in a sample from the subject, of specific binding of any one or more of the invention's antibodies and/or antigen-binding fragments thereof, with the MUC16 polypeptide or with the antigenic portion thereof, wherein detecting an altered level of the specific binding relative to a control sample identifies the subject as having disease. In one embodiment, the disease is cancer exemplified by ovarian cancer and breast cancer. In another embodiment, the method further comprises detecting an altered level of binding of the antibody to the sample compared to a control sample. Optionally, the detecting is selected from the group of immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, immunoprecipitation, and radiographic imaging.

The invention also provides a method for reducing one or more symptoms of disease comprising administering to a subject in need thereof a therapeutically effective amount of any one or more of the invention's antibodies and/or antigen-binding fragment thereof. In one embodiment, the disease is cancer, exemplified by ovarian cancer and breast cancer. Optionally, the method further comprises detecting a reduction in one or more symptoms of the disease after the administration step.

Various aspects of the disclosure relate to an isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is: TLDRSSVLVDGYSPNRNE (SEQ ID NO:2)
wherein the antibody comprises a variable heavy ("V_H") chain encoded by SEQ ID NO:06 and a variable light ("V_L") chain encoded by SEQ ID NO:07.

Various aspects of the disclosure relate to an isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is: TLDRSSVLVDGYSPNRNE (SEQ ID NO:2) wherein the antibody comprises a V_H chain encoded by SEQ ID NO:04 and a V_L chain encoded by SEQ ID NO:05.

Various aspects of the disclosure relate to a humanized antibody or antigen-binding fragment thereof made by substituting the complementarity determining regions of the antibody as described herein into a human framework domain, wherein the humanized antibody or antigen-binding fragment thereof specifically binds to the MUC16 polypeptide of SEQ ID NO:02 or to an antigenic portion thereof.

Various aspects of the disclosure relate to a humanized antibody or antigen-binding fragment thereof made by substituting the complementarity determining regions of the antibody as described herein into a human framework domain, wherein the humanized antibody or antigen-binding fragment thereof specifically binds to the MUC16 polypeptide of SEQ ID NO:02 or to an antigenic portion thereof.

Various aspects of the disclosure relate to a composition comprising (a) an antibody, or antigen-binding fragment thereof, as described herein, and (b) a pharmaceutically acceptable carrier.

Various aspects of the disclosure relate to a hybridoma cell that produces an antibody as described herein.

Various aspects of the disclosure relate to the use of the antibody as described herein, for identifying a subject as having a cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to an *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises (a) contacting a sample obtained from the subject with the antibody as described herein; and (b) determining whether the antibody has an increased level of binding to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to a method for indicating a subject as having disease, comprising determining the level, in a sample from the subject, of specific binding of an antibody of the present invention with a MUC16 polypeptide or with an antigenic portion thereof,

wherein detecting an altered level of the specific binding relative to a control sample indicates the subject as having disease.

Various aspects of the disclosure relate to a single chain variable fragment (scFv) comprising a V_H chain sequence encoded by SEQ ID NO:06 and a V_L chain sequence encoded by SEQ ID NO:07.

Various aspects of the disclosure relate to a chimeric antigen receptor (CAR) comprising the scFv as described herein.

Various aspects of the disclosure relate to a T cell expressing the chimeric antigen receptor (CAR) as described herein.

Various aspects of the disclosure relate to the use of the antibody, or antigen-binding fragment thereof, as described herein, for treating a cancer in a subject, wherein the cancer expresses MUC16.

Various aspects of the disclosure relate to a scFv comprising a VH chain and a VL chain, wherein the VH chain and the VL chain are of a humanized antibody or antigen-binding fragment thereof, wherein the humanized antibody or antigen-binding fragment thereof is made by substituting the complementarity determining regions of an antibody comprising a VH chain encoded by SEQ ID NO:04 and a VL chain encoded by SEQ ID NO:05 into a human framework domain, wherein the humanized antibody or antigen-binding fragment thereof specifically binds to the MUC16 polypeptide of SEQ ID NO:02 or to an antigenic portion thereof.

Various aspects of the disclosure relate to a CAR comprising the scFv as claimed.

Various aspects of the disclosure relate to a CAR comprising the scFv as claimed fused to a transmembrane domain fused to a T cell receptor ζ chain cytoplasmic signaling domain.

Various aspects of the disclosure relate to an isolated antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the MUC16 polypeptide is: CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

Various aspects of the disclosure relate to a composition comprising (a) an antibody, or antigen-binding fragment thereof, as disclosed herein, and (b) a pharmaceutically acceptable carrier.

Various aspects of the disclosure relate to a hybridoma cell that produces an antibody as disclosed herein.

Various aspects of the disclosure relate to a use of the antibody or antigen-binding fragment thereof, as disclosed herein, for identifying a subject as having a cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to an *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises: (a) contacting a sample obtained from the subject with the antibody as disclosed herein; and (b) determining whether the antibody has an increased level of binding to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

Various aspects of the disclosure relate to an isolated polynucleotide comprising a nucleotide sequence that encodes at least one of a variable heavy (VH) chain sequence and the variable light (VL) chain sequence of an antibody that specifically binds to a MUC16 polypeptide, wherein the MUC16 polypeptide is: CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

Various aspects of the disclosure relate to a use of an antibody or antigen-binding fragment thereof, as disclosed herein, for reducing one or more symptoms of a cancer.

Various aspects of the disclosure relate to a use of an antibody or antigen-binding fragment thereof, as disclosed herein, for formulating a medicament for reducing one or more symptoms of a cancer.

Aspects of the disclosure relate to a monoclonal antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is: NFSPLARRVDRVAIYEE (SEQ ID NO:1).

Aspects of the disclosure relate to a single chain variable fragment (scFv) comprising a variable heavy (VH) chain and a variable light (VL) chain, wherein the VH chain and the VL chain are of a monoclonal antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is NFSPLARRVDRVAIYEE (SEQ ID NO:1).

Aspects of the disclosure relate to a hybridoma cell that produces an antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is NFSPLARRVDRVAIYEE (SEQ ID NO:1).

Aspects of the disclosure relate to use of a polypeptide of the sequence: NFSPLARRVDRVAIYEE (SEQ ID NO:1); for producing an antibody or antigen binding fragment thereof that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof.

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Aspects of the disclosure relate to a monoclonal antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is: KSYFSDCQVSTFRSVPNRFIFITGVD SLCNFSPL (SEQ ID NO:15).

Aspects of the disclosure relate to a single chain variable fragment (scFv) comprising a variable heavy (VH) chain and a variable light (VL) chain, wherein the VH chain and the VL chain are of a monoclonal antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is KSYFSDCQVSTFRSVPNRFIFITGVD SLCNFSPL (SEQ ID NO:15).

Aspects of the disclosure relate to a hybridoma cell that produces an antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is KSYFSDCQVSTFRSVPNRFIFITGVD SLCNFSPL (SEQ ID NO:15).

Aspects of the disclosure relate to use of a polypeptide of the sequence: KSYFSDCQVSTFRSVPNRFIFITGVD SLCNFSPL (SEQ ID NO:15); for producing an antibody or antigen binding fragment thereof that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof.

Various embodiments of the claimed invention relate to an isolated monoclonal antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is: CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

Various embodiments of the claimed invention relate to single chain variable fragment (scFv) comprising a variable heavy (VH) chain and a variable light (VL) chain, wherein the VH chain and the VL chain are of a monoclonal antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03).

Various embodiments of the claimed invention relate to an *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises: (a) contacting a sample obtained from the subject with the antibody or antigen binding fragment thereof, of any one of claims 1 to 9; and (b) detecting an increased level of binding of the antibody or antigen binding fragment thereof to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

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Various embodiments of the claimed invention relate to use of a polypeptide of the sequence: CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03); for producing an antibody or antigen binding fragment thereof that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof.

Various embodiments of the claimed antibody or antigen-binding fragment thereof may be useful in diagnosing and/or treating cancer.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Three MUC16 carboxy terminus peptides were synthesized at the MSKCC Microchemistry Core Facility. Polypeptide 1 is near the putative cleavage site, Polypeptide 2 is before the transmembrane, and Polypeptide 3 is the internal peptide, which is inside the transmembrane.

Figure 2: Comparison staining of high-grade serous ovarian carcinomas using OC125 (left panel) and 4H11 (right panel).

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Figure 3: Immunohistochemical scoring of OC125 and 4H11 on tissue microarrays of high-grade ovarian serous carcinoma. Only membranous and/or cytoplasmic staining was considered positive. Score 0: No staining; Score 1: <5% strong or weak; Score 2: 5-50% strong or weak; Score 3: 51-75% strong or 51-100% weak; Score 4: 76-99% strong; Score 5: 100% strong. Figure 3A: OC125 (Score 0); Figure 3B: OC125 (Score 1); Figure 3C: OC125 (Score 2); Figure 3D: OC125 (Score 3); Figure 3E: OC125 (Score 4); Figure 3F: OC125 (Score 5); Figure 3G: 4H11 (Score 0); Figure 3H: 4H11 (Score 1); Figure 3I: 4H11 (Score 2); Figure 3J: 4H11 (Score 3); Figure 3K: 4H11 (Score 4); Figure 3L: 4H11 (Score 5).

Figure 4: Western blot analysis. Figure 4A: Western blot analysis of GST-ΔMUC16^{c114} fusion protein with monoclonal antibodies 9C9.21.5.13 and 4H11.2.5. Figure 4B: Western blot analysis of SKOV3-phrGFP-ΔMUC16^{c114} and SKOV3-phrGFP-ΔMUC16^{c334} protein extract and probed with monoclonal antibodies 9C9.21.5.13 and 4H11.2.5.

Figure 5A: MUC16 carboxy terminus monoclonal antibodies binding affinity on OVCAR3 cells (Panels A-D). Figure 5B: Internalization of radio-labeled 4H11 and OC125 monoclonal antibodies on SKOV3-phrGFP-ΔMUC16^{c334} stable transfected cells.

Figure 6A-D: Comparison staining intensities of OC125 and 4H11 monoclonal antibodies on tissue microarrays containing cancers of the prostate (2A, concordant), lung (2B, discordant), breast (2C, discordant), and pancreas (2D, discordant).

Figure 7: FACS analysis as described in the Material and Methods section was performed with commercial antibodies and MUC16 carboxy terminus monoclonal antibodies on OVCAR3 wt, SKOV3-phrGFP-ΔMUC16^{c114} and SKOV3-phrGFP-ΔMUC16^{c334} stable transfected cell lines.

Figure 8: Nucleotide sequence encoding antibody variable heavy (V_H) chain and antibody variable light (V_L) chain. (A) 4A5 V_H (SEQ ID NO:04), (B) 4A5 V_L (SEQ ID NO:05), (C) 4H11 V_H (SEQ ID NO:06), (D) 4H11 V_L (SEQ ID NO:07), (E) 9B11 V_H (SEQ ID NO:08), (F) 9B11 $V_{L.A}$ (SEQ ID NO:09), (G) 9B11 $V_{L.B}$ (SEQ ID NO:10), (H) 24B3 V_H (SEQ ID NO:11), (I) 24B3 V_L (SEQ ID NO:12).

Figure 9: (A) Homo sapiens MUC16 (GenBank NP_078966) (SEQ ID NO:13), (B) Polypeptide 1 (SEQ ID NO:01), (C) Polypeptide 2 (SEQ ID NO:02), (D) Polypeptide 3 (SEQ ID NO:03), (E) Transmembrane domain (SEQ ID NO:14), (F) Polypeptide 4 (SEQ ID NO:15) containing a cysteine loop polypeptide (SEQ ID NO:19).

Figure 10: Schematic of MUC16 structure.

Figure 11. Design and *in vitro* analysis of MUC-CD targeted CARs. (A) Schematic diagram of the first generation 4H11z and second generation 4H11-28z retroviral vectors. 4H11scFv: MUC16 specific scFv derived from the heavy (V_H) and light (V_L) chain variable regions

of the monoclonal antibody 4H11; CD8: CD8 hinge and transmembrane domains; CD28: CD28 transmembrane and cytoplasmic signaling domains; ζ chain: T cell receptor ζ chain cytoplasmic signaling domain; LTR: long terminal repeat; black box: CD8 leader sequence; grey box: $(Gly_4Ser)_3$ linker; arrows indicate start of transcription. (B) FACS analysis of human T cells retrovirally transduced to express either the 4H11z or 19z1 CAR. (C) 4H11z$^+$ but not 19z1$^+$ T cells expand on 3T3(MUC-CD/B7.1) AAPC. CAR$^+$ were co-cultured on 3T3(MUC-CD/B7.1) AAPC monolayers at 3 x 10^6 CAR$^+$ T cells/well of a 6 well plate. Proliferation of CAR$^+$ T cells, normalized to the CAR$^+$ T cell fraction as assessed by FACS for the CAR$^+$ fraction in combination with viable T cell counts obtained on days 2, 4 and 7, as assessed by trypan blue exclusion assays.

Figure 12. *In vitro* comparison of T cells modified to express the first generation 4H11z CAR to T cells modified to express the second generation co-stimulatory 4H11-28z CAR. (A) CAR$^+$ T cells were co-cultured on MUC-CD monolayers with (right panel) or without B7.1 (left panel). 3 x 10^6 CAR$^+$ T cells were co-cultured on AAPC monolayers in 6 well tissue culture plates in cytokine-free medium. Total viable T cell counts were assessed on days 2, 4 and 7, by trypan blue exclusion assays. 4H11-28z$^+$ T cells markedly expanded when compared to 4H11z$^+$ T cells upon co-culture with 3T3(MUC-CD) AAPCs, **p=0.0023 (4H11z compared to 4H11-28z). In contrast, both 4H11z$^+$ and 4H11-28z$^+$ T cells expanded similarly on 3T3(MUC-CD/B7.1) AAPCs, p=0.09, (4H11z compared to 4H11-28z). Control 19-28z$^+$ T cells did not proliferate on 3T3(MUC-CD), **p=0.0056 (19-28z compared to 4H11z), **p=0.0011 (19-28z compared to 4H11-28z), or on 3T3(MUC-CD/B7.1), **p=0.0026 (19-28z compared to 4H11z), **p=0.0087 (19-28z compared to 4H11-28z). (B) 4H11-28z$^+$ but not 4H11z$^+$ T cells secrete IL-2 upon co-culture with 3T3(MUC-CD) AAPCs. Tissue culture supernatants at day 2 following activation on 3T3(MUC-CD) AAPCs were analyzed for cytokine secretion. 4H11-28z$^+$ T cells, in contrast to 4H11z$^+$ T cells, demonstrated enhanced secretion of IL-2 consistent with T cell co-stimulation mediated through the 4H11-28z CAR. ***p=0.0008 (19z1 or 19-28z compared to 4H11z), **p=0.0026 (19z1 or 19-28z compared to 4H11-28z), **p=0.0046 (4H11z compared to 4H11-28z). Furthermore, both 4H11-28z$^+$ and 4H11z$^+$ T cells secreted IFNγ. *p=0.011 (4H11z compared to 4H11-28z). Control 19z1 and 1928z transduced T cells failed to secrete either IL-2 or IFNγ. **p=0.0034 (19z1 compared to 4H11z), **p=0.036 (19-28z compared to 4H11z), ***p=0.0008 (19-28z compared to 4H11-28z). (C) Expansion of CAR$^+$ T cells following 3 cycles of stimulation on 3T3(MUC-CD/B7.1). Human T cells transduced to express either 4H11z or 4H11-28z CARs demonstrated a >2 log expansion over 2 cycles of stimulation on 3T3(MUC-CD/B7.1) AAPCs. Arrows indicate 1st and 2nd cycles of restimulation on AAPCs. (D) FACS analysis of the CAR$^+$ T cell fraction of 4H11-28z$^+$ T cells increased following each weekly cycle of stimulation. (I) FACS following initial transduction, (II)

FACS at 7 days following first stimulation on AAPCs, (III) FACS at 7 days following second stimulation on AAPCs. These data are representative of one of three different experiments using three different healthy donor T cell populations, all of which demonstrated similar proliferation and cytokine secretion patterns.

Figure 13. MUC-CD targeted T cells specifically expand and lyse MUC-CD$^+$ tumor cells. (A) Cytotoxicity assay of 4H11z$^+$ and 4H11-28z$^+$ T cells targeting OV-CAR(MUC-CD) tumor cells demonstrates efficient cytotoxicity mediated by T cells from healthy donors modified to express the first and second generation MUC-CD targeted CARs. Control T cells modified to express the first and second generation CD19-targeted 19z1 and 19-28z CARs failed to demonstrate significant lysis of target tumor cells. (B) Healthy donor T cells modified to express the 4H11-28z CAR equally lyse primary patient ascites-derived MUC-CD$^+$ tumor cells when compared to T cells modified to express the control 19-28z CAR. This data represents 1 or 3 experiments targeting primary tumor cells from 3 ovarian carcinoma patients with similar results. (C) Autologous T cells isolated from peripheral blood, when modified with the 4H11-28z CAR, exhibit significant lysis of autologous MUC-CD$^+$ ascites-derived tumor cells when compared to control 19-28z$^+$ autologous T cells. These data represent 1 of 3 experiments utilizing T cells and autologous tumor cells from 3 different ovarian carcinoma patients with similar results. (D) Antigen specific proliferation of MUC-CD targeted CFSE labeled T cells after co-culture with OV-CAR3(MUC-CD) tumor cells. CFSE labeled CAR$^+$ T cells were co-cultured with MUC-CD expressing OV-CAR3 tumor cells at 1:1 ratio for 5 days. Proliferation of CFSE labeled T cells was assessed by FACS demonstrating efficient proliferation of both 4H11z$^+$ and 4H11-28z$^+$ T cells but not control 19-28z$^+$ T cells. (E) CFSE results were further confirmed by absolute T cell numbers assessed on days 2, 4 and 7 following co-culture with OV-CAR3(MUC-CD) tumor cells. (F) FACS analysis of the expression of 4-1BBL on OVCAR3(MUC-CD) cells. OV-CAR3(MUC-CD) cells were stained with anti-human 4-1BBL antibody (thick line) or with isotype control (thin line). FACS analysis demonstrated expression of 4-1BBL on OV-CAR3(MUC-CD) tumor cells. Further FACS analyses failed to reveal expression of the co-stimulatory ligands B7.1, B7.2, or OX-40L.

Figure 14. Eradication of OV-CAR3(MUC-CD) tumors after intra-peritoneal treatment with first and second generation of MUC-CD targeted T cells. (A) Intraperitoneal injection of OV-CAR3(MUC-CD) tumors in untreated SCID-Beige mice results in abdominal distension and nodular peritoneal tumors. SCID-Beige mice were injected intraperitoneally with 3x10^6 OV-CAR3(MUC-CD) cells. At 5 weeks post intraperitoneal injection of OV-CAR3(MUC-CD) tumor cells mice developed ascities as evidenced by a distended abdomen (center panel) when compared to a tumor free mouse (left panel). Post mortem visualization of the peritoneum demonstrates

nodular tumor masses (arrows) within the abdominal cavity (right panel). (B) Intraperitoneal injection of 4H11z$^+$ and 4H11-28z$^+$ T cells either delay tumor progression or fully eradicate disease. Kaplan-Meier survival curve of SCID-Beige mice treated with first or second generation of MUC-CD targeted T cells. SCID-Beige mice were infused ip with 3x10^6 OV-CAR3(MUC-CD) tumor cells on day 1 followed by 3x10^7 4H11z$^+$ or 4H11-28z$^+$ T cells on day 2. All untreated mice or mice treated with control 19z1$^+$ T cells developed established tumors and were sacrificed by day 50. In contrast, 27% of mice treated with either 4H11z$^+$ or 4H11-28z$^+$ T cells remained without clinical evidence of disease by day 120. *p=0.01 (4H11z compared to 19z1), **p=0.0023 (4H11-28z compared to 19z1), p=0.63 (4H11z compared to 4H11-28z).

Figure 15. MUC-CD targeted 4H11-28z$^+$ T cells successfully traffic to ip OV-CAR3(MUC-CD/GFP-FFLuc) tumors following systemic intravenous infusion resulting in equally efficient anti-tumor efficacy when compared to ip 4H11-28z$^+$ treated tumor bearing mice. (A) Kaplan-Meier survival curve of SCID-Beige mice treated ip or iv with 4H11-28z$^+$ T cells. SCID-Beige mice were injected intraperitoneally with 3x10^6 OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells followed by either iv or ip infusion of 3x10^7 4H11-28z$^-$ T cells. Tumor eradication is enhanced after either ip or iv infusion of 4H11-28z$^+$ T cells when compared to control treated mice. Both ip and iv 4H11-28z$^+$ T cell treated mice exhibited statistically enhanced survival (***p<0.0001 and **p=0.0038, respectively) when compared to 19-28z$^+$ T cell treated control cohorts. Conversely, difference in survival between the ip and iv 4H11-28z$^+$ T cell cohorts was not statistically significant (p=0.22). (B) BLI of tumor progression of representative ip and iv 4H11-28z$^+$ T cell treated mice with ultimately progressive disease following treatment compared to BLI of tumor progression in a representative control 19-28z$^+$ T cell treated mouse. (C) Systemically injected CFSE stained 4H11-28z$^+$ T cells traffic to advanced ip OV-CAR(MUC-CD) tumors. Presence of iv injected CFSE labeled 19-28z$^+$ control T cells (left panel) and 4H11-28z$^+$ T cells (right panel) 1 day following infusion into SCID-Beige mice with advanced OV-CAR(MUC-CD) tumors (injected 7 days earlier), as assessed by FACS analysis of single cell OV-CAR3(MUC-CD) tumor suspensions, reveals a marked population of 4H11-28z$^+$ but not control 19-28z$^+$ T cells within peritoneal OV-CAR3(MUC-CD) tumors.

Figure 16. Eradication of advanced OV-CAR3(MUC-CD) tumors in SCID-Beige mice by ip infusion of 4H11-28z$^+$ T cells. SCID-Beige mice were injected ip with 3x10^6 OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells 7 days prior to ip treatment with 3x10^7 4H11-28z$^+$ T cells. (A) BLI of 4H11-28z$^+$ T cell treated mice with either relapsed disease (middle row) or eradicated disease (bottom row) compared to a representative 19-28z$^+$ T cell treated control mouse. (B) Kaplan-Meier survival curve of SCID-Beige mice with advanced OV-CAR3(MUC-CD/GFP-FFLuc) tumors

treated ip with 4H11-28z$^+$ T cells. All 4H11-28z$^+$ T cell treated mice demonstrated enhanced survival when compared to control 19-28z$^+$ T cell treated mice (**p=0.0011), with an overall long-term survival of 25% at day 120.

Figure 17: CD8 leader sequence, CD3 zeta chain intracellular domain sequence, (G4S)3 serine-glycine linker sequence, CD8 transmembrane domain sequence, and CD28 transmembrane + intracellular domains (-STOP) sequence.

Figure 18: SFG_4H11z sequence.

Figure 19: SFG-4H11-28z sequence.

Figure 20: (A) Mouse MUC16-CD Peptide 1 (SEQ ID NO:21), Mouse first Cysteine Loop Peptide 2 (SEQ ID NO:22), and Mouse second Cysteine Loop Peptide 3 (SEQ ID NO:23). (B) Alignment of mouse MUC16 (SEQ ID NO:24) and human MUC16 (SEQ ID NO:25) amino acid sequences. A cysteine was added to the peptide sequence at the N terminus of Peptide 1 and Peptide 3 for better conjugation with KLH.

Figure 21: ID8 extract with 1:10 dilution of Mouse MUC16 monoclonal Primary Supernatants.

Figure 22: BR5-FVB1 extract with 1:10 dilution of Mouse MUC16 monoclonal Primary Supernatants

Figure 23: Western Blot showing 38 hamster's monoclonal antibody Supernatants on ID8 cell extracts.

Figure 24 (A) Nucleotide sequence encoding 12B10-3G10-V_H (SEQ ID NO:26), (B) 12B10-3G10-V_H Amino Acid sequence (SEQ ID NO:27), (C) Nucleotide sequence encoding 12B10-3G10-V_L (SEQ ID NO:28) (Note the VL has an optional *NotI* site added by the primer for cloning, and (D) 12B10-3G10-V_L Amino Acid sequence (SEQ ID NO:29).

Figure 25: FACS Analysis with Purified 12B10-3G10 mAb on ID8 (mouse), OVCAR-3 (human) and BR5-FVB1 (mouse) cell lines.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The terms "purified," "isolated," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one undesirable component (such as cell, protein, nucleic acid sequence, carbohydrate, *etc.*) from a sample, including a reduction by any numerical percentage of from 5% to 100%, such as, but not limited to, from 10% to 100%, from 20% to 100%, from 30% to 100%, from 40% to 100%, from 50% to 100%, from 60% to 100%, from 70% to 100%, from 80% to 100%, and from 90% to 100%. Thus purification results in an "enrichment,"

i.e., an increase in the amount of a desirable component cell, protein, nucleic acid sequence, carbohydrate, *etc.*).

The term "antibody" refers to an immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, *etc.*). The basic functional unit of each antibody is an immunoglobulin (Ig) mononer (containing only one immunoglobulin ("Ig") unit). Included within this definition are polyclonal antibody, monoclonal antibody, and chimeric antibody.

The variable part of an antibody is its "V domain" (also referred to as "variable region"), and the constant part is its "C domain" (also referred to as "constant region") such as the kappa, lambda, alpha, gamma, delta, epsilon and mu constant regions. The "variable domain" is also referred to as the "F_V region" and is the most important region for binding to antigens. More specifically, variable loops, three each on the light (V_L) and heavy (V_H) chains are responsible for binding to the antigen. These loops are referred to as the "complementarity determining regions" ("CDRs" and "idiotypes."

The immunoglobulin (Ig) monomer of an antibody is a "Y"-shaped molecule that contains four polypeptide chains: two light chains and two heavy chains, joined by disulfide bridges.

Light chains are classified as either (λ) or kappa (κ). A light chain has two successive domains: one constant domain ("C_L") and one variable domain ("V_L"). The variable domain, V_L, is different in each type of antibody and is the active portion of the molecule that binds with the specific antigen.The approximate length of a light chain is 211 to 217 amino acids.

Each heavy chain has two regions, the *constant region* and the *variable region*. The There are five types of mammalian Ig heavy denoted a α, δ, ε, γ, and μ. The type of heavy chain present defines the *class* of antibody; these chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively. Distinct heavy chains differ in size and composition; α and γ contain approximately 450 amino acids, while μ and ε have approximately 550 amino acids. Each heavy chain has two regions, the constant region ("C_H") and the variable ("V_H") region. The constant region (C_H) is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. Heavy chains γ, α and δ have a constant region composed of *three* tandem (in a line) Ig domains, and a hinge region for added flexibility. Heavy chains μ and ε have a constant region composed of *four* immunoglobulin domains. The variable region (V_H) of the heavy chain differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone. The variable region of each heavy chain is approximately 110 amino acids long.

The term "specifically binds" and "specific binding" when made in reference to the binding of two molecules (*e.g.* antibody to an antigen, *etc.*) refer to an interaction of the two molecules that is dependent upon the presence of a particular structure on one or both of the molecules. For

example, if an antibody is specific for epitope "A" on the molecule, then the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

The term "capable of binding" when made in reference to the interaction between a first molecule (such as antibody, polypeptide, glycoprotein, nucleic acid sequence, *etc.*) and a second molecule (such as antigen, polypeptide, glycoprotein, nucleic acid sequence, *etc.*) means that the first molecule binds to the second molecule in the presence of suitable concentration of salts, and suitable temperature, and pH. The conditions for binding molecules may be determined using routine and/or commercially available methods

The terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," "immunologic," and "immunologically active" when made in reference to a molecule, refer to any substance that is capable of inducing a specific humoral immune response (including eliciting a soluble antibody response) and/or cell-mediated immune response (including eliciting a CTL response). Antigenic peptides preferably contain at least 5, at least 6, at least 7, at least 8, at least 9, and more preferably at least 10 amino acids. To elicit antibody production, in one embodiment, antigens may be conjugated to keyhole limpet hemocyanin (KLH) or fused to glutathione-S-transferase (GST).

A "cognate antigen" when in reference to an antigen that binds to an antibody, refers to an antigen that is capable of specifically binding to the antibody.

In one embodiment, the antigen comprises an epitope. The terms "epitope" and "antigenic determinant" refer to a structure on an antigen, which interacts with the binding site of an antibody or T cell receptor as a result of molecular complementarity. An epitope may compete with the intact antigen, from which it is derived, for binding to an antibody.

As used herein the terms "portion" and "fragment" when made in reference to a nucleic acid sequence or protein sequence refer to a piece of that sequence that may range in size from 2 contiguous nucleotides and amino acids, respectively, to the entire sequence minus one nucleotide and amino acid, respectively.

A "subject" that may benefit from the invention's methods includes any multicellular animal, preferably a mammal. Mammalian subjects include humans, non-human primates, murines, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, *etc.*). Thus, mammalian subjects are exemplified by mouse, rat, guinea pig, hamster, ferret and chinchilla. The invention's compositions and methods are also useful for a subject "in need of reducing one or more symptoms of" a disease, *e.g.*, in need of reducing cancer metastasis and/or in need of reducing one or more symptoms of cancer, includes a subject that exhibits and/or is at risk

of exhibiting one or more symptoms of the disease. For Example, subjects may be at risk based on family history, genetic factors, environmental factors, *etc.* This term includes animal models of the disease. Thus, administering a composition (which reduces a disease and/or which reduces one or more symptoms of a disease) to a subject in need of reducing the disease and/or of reducing one or more symptoms of the disease includes prophylactic administration of the composition (i.e., before the disease and/or one or more symptoms of the disease are detectable) and/or therapeutic administration of the composition (i.e., after the disease and/or one or more symptoms of the disease are detectable). The invention's compositions and methods are also useful for a subject "at risk" for disease (such as cancer) refers to a subject that is predisposed to contracting and/or expressing one or more symptoms of the disease. This predisposition may be genetic (*e.g.,* a particular genetic tendency to expressing one or more symptoms of the disease, such as heritable disorders, etc.), or due to other factors (*e.g.,* environmental conditions, exposures to detrimental compounds, including carcinogens, present in the environment, *etc.*). The term subject "at risk" includes subjects "suffering from disease," i.e., a subject that is experiencing one or more symptoms of the disease. It is not intended that the present invention be limited to any particular signs or symptoms. Thus, it is intended that the present invention encompass subjects that are experiencing any range of disease, from sub-clinical symptoms to full-blown disease, wherein the subject exhibits at least one of the indicia (*e.g.,* signs and symptoms) associated with the disease.

"Cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described (Pitot et al., Fundamentals of Oncology, 15-28 (1978)). This includes cells in early, intermediate and advanced stages of neoplastic progression including "pre-neoplastic cells (i.e., "hyperplastic cells and dysplastic cells), and neoplastic cells in advanced stages of neoplastic progression of a dysplastic cell.

"Metastatic" cancer cell refers to a cancer cell that is translocated from a primary cancer site (*i.e.*, a location where the cancer cell initially formed from a normal, hyperplastic or dysplastic cell) to a site other than the primary site, where the translocated cancer cell lodges and proliferates.

"Cancer" refers to a plurality of cancer cells that may or may not be metastatic, such as ovarian cancer, breast cancer, lung cancer, prostate cancer, cervical cancer, pancreatic cancer, colon cancer, stomach cancer, esophagus cancer, mouth cancer, tongue cancer, gum cancer, skin cancer (e.g., melanoma, basal cell carcinoma, Kaposi's sarcoma, etc.), muscle cancer, heart cancer, liver cancer, bronchial cancer, cartilage cancer, bone cancer, testis cancer, kidney cancer, endometrium cancer, uterus cancer, bladder cancer, bone marrow cancer, lymphoma cancer, spleen cancer, thymus cancer, thyroid cancer, brain cancer, neuron cancer, mesothelioma, gall bladder cancer, ocular cancer (e.g., cancer of the cornea, cancer of uvea, cancer of the choroids, cancer of the

macula, vitreous humor cancer, etc.), joint cancer (such as synovium cancer), glioblastoma, lymphoma, and leukemia.

"Sample" and "specimen" as used herein are used in their broadest sense to include any composition that is obtained and/or derived from a biological source, as well as sampling devices (*e.g.*, swabs), which are brought into contact with biological or environmental samples. "Biological samples" include those obtained from a subject, including body fluids (such as urine, blood, plasma, fecal matter, cerebrospinal fluid (CSF), semen, sputum, and saliva), as well as solid tissue. Biological samples also include a cell (such as cell lines, cells isolated from tissue whether or not the isolated cells are cultured after isolation from tissue, fixed cells such as cells fixed for histological and/or immunohistochemical analysis), tissue (such as biopsy material), cell extract, tissue extract, and nucleic acid (e.g., DNA and RNA) isolated from a cell and/or tissue, and the like. These examples are illustrative, and are not to be construed as limiting the sample types applicable to the present invention.

"Overexpression of MUC16" by a cell of interest (such as a cancer cell) refers to a higher level of MUC16 protein and/or mRNA that is expressed by the cell of interest compared to a control cell (such as a non-cancerous cell, normal cell, *etc.*).

"Internalize" when in reference to a cell refers to entry from the extracellular medium into the cell membrane and/or cytoplasm.

"Glycosylated" when in reference to a sequence (e.g., an amino acid sequence or nucleotide sequence) refers to a sequence that is covalently linked to one or more saccharides.

"Pharmaceutical" and "physiologically tolerable " composition refers to a composition that contains pharmaceutical molecules, *i.e.*, molecules that are capable of administration to or upon a subject and that do not substantially produce an undesirable effect such as, for example, adverse or allergic reactions, dizziness, gastric upset, toxicity and the like, when administered to a subject. Preferably also, the pharmaceutical molecule does not substantially reduce the activity of the invention's compositions. Pharmaceutical molecules include "diluent" (i.e., "carrier") molecules and excipients.

"Immunogenically effective" and "antigenically effective" amount of a molecule interchangeably refer to an amount of the molecule that is capable of inducing a specific humoral immune response (including eliciting a soluble antibody response) and/or cell-mediated immune response (including eliciting a cytotoxic T-lymphocyte (CTL) response).

"Treating" a disease refers to reducing one or more symptoms (such as objective, subjective, pathological, clinical, sub-clinical, etc.) of the disease.

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents (including "lower," "smaller," *etc.*) when in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, antibody, etc.), cell, and/or phenomenon (*e.g.*, disease symptom, binding to a molecule, specificity of binding of two molecules, affinity of binding of two molecules, specificity to cancer, sensitivity to cancer, affinity of binding, enzyme activity, *etc.*) in a first sample (or in a first subject) relative to a second sample (or relative to a second subject), mean that the quantity of molecule, cell and/or phenomenon in the first sample (or in the first subject) is lower than in the second sample (or in the second subject) by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the quantity of molecule, cell and/or phenomenon in the first sample (or in the first subject) is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). In another embodiment, the quantity of molecule, cell, and/or phenomenon in the first sample (or in the first subject) is lower by any numerical percentage from 5% to 100%, such as, but not limited to, from 10% to 100%, from 20% to 100%, from 30% to 100%, from 40% to 100%, from 50% to 100%, from 60% to 100%, from 70% to 100%, from 80% to 100%, and from 90% to 100% lower than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). In one embodiment, the first subject is exemplified by, but not limited to, a subject that has been manipulated using the invention's compositions and/or methods. In a further embodiment, the second subject is exemplified by, but not limited to, a subject that has not been manipulated using the invention's compositions and/or methods. In an alternative embodiment, the second subject is exemplified by, but not limited to, a subject to that has been manipulated, using the invention's compositions and/or methods, at a different dosage and/or for a different duration and/or via a different route of administration compared to the first subject. In one embodiment, the first and second subjects may be the same individual, such as where the effect of different regimens (e.g., of dosages, duration, route of administration, etc.) of the invention's compositions and/or methods is sought to be determined in one individual. In another embodiment, the first and second subjects may be different individuals, such as when comparing the effect of the invention's compositions and/or methods on one individual participating in a clinical trial and another individual in a hospital.

The terms "increase," "elevate," "raise," and grammatical equivalents (including "higher," "greater," *etc.*) when in reference to the level of any molecule (*e.g.*, amino acid sequence, and nucleic acid sequence, antibody, etc.), cell, and/or phenomenon (*e.g.*, disease symptom, binding to a molecule, specificity of binding of two molecules, affinity of binding of two molecules,

specificity to cancer, sensitivity to cancer, affinity of binding, enzyme activity, *etc.*) in a first sample (or in a first subject) relative to a second sample (or relative to a second subject), mean that the quantity of the molecule, cell and/or phenomenon in the first sample (or in the first subject) is higher than in the second sample (or in the second subject) by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the quantity of the molecule, cell and/or phenomenon in the first sample (or in the first subject) is at least 10% greater than, at least 25% greater than, at least 50% greater than, at least 75% greater than, and/or at least 90% greater than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). This includes, without limitation, a quantity of molecule, cell, and/or phenomenon in the first sample (or in the first subject) that is at least 10% greater than, at least 15% greater than, at least 20% greater than, at least 25% greater than, at least 30% greater than, at least 35% greater than, at least 40% greater than, at least 45% greater than, at least 50% greater than, at least 55% greater than, at least 60% greater than, at least 65% greater than, at least 70% greater than, at least 75% greater than, at least 80% greater than, at least 85% greater than, at least 90% greater than, and/or at least 95% greater than the quantity of the same molecule, cell and/or phenomenon in the second sample (or in the second subject). In one embodiment, the first subject is exemplified by, but not limited to, a subject that has been manipulated using the invention's compositions and/or methods. In a further embodiment, the second subject is exemplified by, but not limited to, a subject that has not been manipulated using the invention's compositions and/or methods. In an alternative embodiment, the second subject is exemplified by, but not limited to, a subject to that has been manipulated, using the invention's compositions and/or methods, at a different dosage and/or for a different duration and/or via a different route of administration compared to the first subject. In one embodiment, the first and second subjects may be the same individual, such as where the effect of different regimens (e.g., of dosages, duration, route of administration, etc.) of the invention's compositions and/or methods is sought to be determined in one individual. In another embodiment, the first and second subjects may be different individuals, such as when comparing the effect of the invention's compositions and/or methods on one individual participating in a clinical trial and another individual in a hospital.

The terms "alter" and "modify" when in reference to the level of any molecule and/or phenomenon refer to an increase or decrease.

Reference herein to any numerical range expressly includes each numerical value (including fractional numbers and whole numbers) encompassed by that range. To illustrate, and without limitation, reference herein to a range of "at least 50" includes whole numbers of 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, *etc.*, and fractional numbers 50.1, 50.2 50.3, 50.4, 50.5, 50.6, 50.7, 50.8,

50.9, *etc.* In a further illustration, reference herein to a range of "less than 50" includes whole numbers 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, *etc.*, and fractional numbers 49.9, 49.8, 49.7, 49.6, 49.5, 49.4, 49.3, 49.2, 49.1, 49.0, *etc.* In yet another illustration, reference herein to a range of from "5 to 10" includes each whole number of 5, 6, 7, 8, 9, and 10, and each fractional number such as 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, *etc.*

DESCRIPTION OF THE INVENTION

The invention provides antibodies, and antigen-binding fragments thereof, that specifically bind to a polypeptide, or antigenic portion thereof, wherein the polypeptide is selected from a) MUC16 ectodomain polypeptide, b) MUC16 cytoplasmic domain polypeptide, and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide. The invention's antibodies and compositions containing them are useful in diagnostic and therapeutic applications for diseases in which MUC16 is overexpressed, such as cancer.

Using synthetic peptides, the inventors raised novel-specific antibodies to the carboxy-terminal portion of MUC16, retained by the cell, proximal to the putative cleavage site. These antibodies were characterized using fluorescence-activated cell-sorting analysis, enzyme-linked immunoassay, Western blot analysis, and immunohistochemistry. Each of the selected monoclonal antibodies was reactive against recombinant GST-ΔMUC16^{c114} protein and the MUC16 transfected SKOV3 cell line. Three antibodies, 4H11, 9C9, and 4A5 antibodies demonstrated high affinities by Western blot analysis and saturation-binding studies of transfected SKOV3 cells, and displayed antibody internalization. Immunohistochemical positivity with novel antibody 4H11 was similar to OC125, but with important differences, including diffuse positivity in lobular breast cancer and a small percentage of OC125-negative ovarian carcinomas which showed intense and diffuse 4H11 antibody binding.

The invention's compositions and methods are useful for diagnostic and therapeutic applications, as well as biologic studies such as membrane receptor trafficking and intracellular events. Diagnostic applications include, for example, detection of cancer using immunohistochemical, radiographic imaging, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, and/or immunoprecipitation detection.

The invention is further described under (A) MUC16, (B) Prior Art Antibodies, (C) Invention's Antibodies, (D) Hybridoma Cell Lines, (E) Conjugates Of The Invention's Antibodies Linked To Cytotoxic Agents And/Or Prodrugs, (F) Detecting Muc16 Portions And Diagnostic Applications, and (G) Therapeutic Applications.

A. MUC16

"MUC16," "MUC-16" and "Mucin 16" interchangeably refer to a type I membrane protein that is part of a family of tethered mucins. A schematic of Muc16 is in Figure 10, and an exemplary human Muc16 amino acid sequence (SEQ ID NO:13) is shown in Figure 9A. An alignment of mouse MUC16 (SEQ ID NO:24) and human MUC16 (SEQ ID NO:25) amino acid sequences is shown in Figure 20B. The term "type 1 protein" refers to a "membrane protein" that is at least partially embedded in the lipid bilayer of a cell, virus and the like, and that contains a transmembrane domain (TM) sequence embedded in the lipid bilayer of the cell, virus and the like. The portion of the protein on the NH_2-terminal side of the TM domain is exposed on the exterior side of the membrane, and the COOH-terminal portion is exposed on the cytoplasmic side.

Recently, the sequence of the cDNA-encoding MUC16/CA125 was described by Yin and Lloyd in 2001 and completed by O'Brien in 2002 (10-12). The complete MUC16 protein has various components consisting of a cytoplasmic tail with potential phosphorylation sites, a transmembrane domain, and an external domain proximal to an apparent cleavage site. Distal to the cleavage site, the released external domain contains 16-20 tandem repeats of 156 amino acids, each with many potential glycosylation sites (11). The overall repeat structure (Figure 10) is well conserved across mammals, but the repeats are not completely identical in exact amino acid composition.

The MUC16 protein is part of a family of tethered mucins that includes both MUC1 and MUC4 (13). MUC1 is present in a variety of tissues and appears to signal through a beta catenin pathway, interact with EGF receptor, mediates drug resistance and can act as an oncogene (14-17). The MUC4 protein is also expressed in a variety of tissues but is common on neoplasms of the gastrointestinal track (18-20). In contrast, the CA125 antigen has been more restricted in its distribution and is present primarily in gynecologic tissues and overexpressed in Müllerian neoplasms (21). However, the CA125 antigen, recognized by the OC125 antibody, is a heavily glycosylated antigen expressed in the tandem repeat region of the larger MUC16 protein. This glycoprotein is typically shed from a putative cleavage site in the extracellular domain of the MUC16 peptide backbone.

Thus, "MUC16" protein contains (a) a "cytoplasmic domain," (b)a "transmembrane domain," and (c) a "extracellular domain." The MUC16 extracellular domain contains a cleavage site between a non-glycosylated ectodomain and a large glycosylated ectodomain of tandem repeats.

The terms "cytoplasmic domain," "cytoplasmic tail," and "CT" are used interchangeably to refer to a protein sequence, and portions thereof, that is on the cytoplasmic side of the lipid bilayer

of a cell, virus and the like. Methods for determining the CT of a protein are known in the art Elofsson et al. (2007) Annu. Rev. Biochem. 76:125-140; Bernsel et al. (2005) Protein Science 14:1723-1728).

The terms "transmembrane domain" and "TM" are used interchangeably to refer to a protein sequence, and portions thereof, that spans the lipid bilayer of a cell, virus and the like. Methods for determining the TM of a protein are known in the art (Elofsson et al. (2007) Annu. Rev. Biochem. 76:125-140; Bernsel et al. (2005) Protein Science 14:1723-1728).

The terms "ectodomain" and "extracellular domain" are interchangeably used when in reference to a membrane protein to refer to the portion of the protein that is exposed on the extracellular side of a lipid bilayer of a cell, virus and the like. Methods for determining the ectodomain of a protein are known in the art (Singer (1990) Annu. Rev. Cell Biol. 6:247-296 and High et al. (1993) J. Cell Biol. 121:743-750, and McVector software, Oxford Molecular).

The exemplary Muc16 of Figure 9 contains (a) a "MUC16 cytoplasmic domain" from amino acid 14476 to 14507, vttrr rkkegeynvq qqcpgyyqsh ldledlq (SEQ ID NO:16), that interacts with the intracellular signal transduction machinery; (b) a "MUC16 transmembrane domain" from amino acid 14452 to 14475, fwaviligl agllgvitcl icgvl (SEQ ID NO:14) that spans the plasma membrane; and (c) a "MUC16 extracellular domain" amino acid 1 to 14392 (SEQ ID NO:13) that contains a cleavage site between an non-glycosylated ectodomain and a large glycosylated ectodomain of tandem repeats. The "MUC16 ectodomain" is exemplified by nfsplar rvdrvaiyee flrmtrngtq lqnftldrss vlvdgyspnr nepltgnsdl p (SEQ ID NO:17) from amino acid 14394 to 14451 of SEQ ID NO:13 of Figure 9A.

The exemplary MUC16 ectodomain contains both Polypeptide 1 (nfsplar rvdrvaiyee (SEQ ID NO:01), which is from amino acid 14394 to 14410 of SEQ ID NO:13), and Polypeptide 2 (tldrss vlvdgyspnr ne (SEQ ID NO:02), which is from amino acid 14425 to 14442 of SEQ ID NO:13), against which the invention's exemplary antibodies were produced. Polypeptide 3, cgvlvttrr rkkegeynvq qq (SEQ ID NO:03) is from amino acid 14472 to 14492 of SEQ ID NO:13, and contains both a transmembrane domain portion (cgvl) and a cytoplasmic domain portion (vttrr rkkegeynvq qq (SEQ ID NO:18)). Thus, the CGVL is optional in SEQ ID NO:03, as it is part of the transmembrane domain.

Polypeptide 4 (ksyf sdcqvstfrs vpnrhhtgvd slcnfspl (SEQ ID NO:15), is located in a non-glycosylated portion of the Muc16 extracellular domain, is from amino acid 14367 to 14398 of SEQ ID NO:13, and contains a cysteine loop polypeptide cqvstfrsvpnrhhtgvdslc (SEQ ID NO:13).

B. Prior Art Antibodies

The expression of the MUC16/CA125 antigen has long been associated with gynecologic tissues. "CA125," "CA-125," "Cleaved CA125," and "cleaved CA-125," interchangeably refer to the glycosylated external domain of the tethered mucin MUC16, that is distal to the cleavage site (Payne et al., U.S. Pat. No. 7,202,346). This released external domain contains 16-20 tandem repeats of 156 amino acids, each with potential glycosylation sites. An apparent cysteine-based disulfide loop of 19 amino acids is present in all repeats and the N-terminal end contains a hairbrush structure that is heavily O-glycosylated (11). The deduced size would be 2.5 MD for the protein part, and with added carbohydrates, this could increase to 5 MD (10, 26).

CA125, though it is not sensitive or specific enough to be used as a general screening tool, is routinely used to monitor patients with ovarian carcinoma. The tests used to measure CA125 are antibody based detection methods, as are the immunohistochemical stains routinely performed for diagnostic purposes. The epitope specificity of 26 antibodies to MUC16 was studied in the first report from the International Society of Oncodevelopmental Biology and Medicine (ISOBM) TD-1 Workshop and the application of 22 antibodies to immunohistochemistry was reported in the second report from the TD-1 workshop (7, 21). The existing antibodies were grouped as OC125-like, M11-like, or OV197-like and all of the known antibodies recognized CA125 epitopes in the repeating, glycosylated elements in the external domain of the tethered mucin MUC16, distal to the putative cleavage site.

The vast majority of MUC16-reactive antibodies, including OC125, react with the glycosylation-dependent antigen present exclusively in the cleaved portion of the molecule so the true distribution of MUC16 expression is not known (21). There is currently no antibody available to track the fate of the remaining MUC16 protein fragment after cleavage and CA125 release.

C. Invention's Antibodies

In order to better explore the biology of human MUC16, the inventors have derived monoclonal antibodies against the extracellular portion of the MUC16-carboxy terminus, proximal to the putative cleavage site, as well as one monoclonal antibody against the internal cytoplasmic domain. In contrast to prior antibodies, these are derived against the peptide backbone of MUC16 and are not directed at complex glycoprotein epitopes. Since these epitopes are proximal to the cleavage site, they are unlikely to be found in the circulation and provide novel targets for diagnostic methods and therapeutic interventions. Data herein demonstrate the identification and characterization of exemplary antibodies developed against the MUC16 peptide backbone.

The inventors have developed novel antibodies that are directed at the non-cleaved, non-glycosylated peptide backbone of MUC16. These are exemplified by both 4H11 and 9C9 antibodies, which react with peptide sequences in the non-cleaved ectodomain of MUC16 and are detectable on the surface of ovarian cancer cell lines and in paraffin-fixed tissues from human ovarian cancer surgical specimens. The antibodies show high affinity and are readily internalized by ovarian cancer cells when bound to the ectodomain of MUC16. This suggests that the proximal portion of MUC16 has an independent biology from the more distal, cleaved portion of the mucin. It also suggests that the proximal portions of MUC16 could provide convenient targets for diagnostic and therapeutic interventions. Targeting the peptide backbone of MUC16 provides highly specific tissue delivery for genetically engineered cells, liposomes, or antibody conjugates, including conjugates with the invention's antibodies.

The invention's antibodies, exemplified by antibody 4H11, are useful as tools in immunohistochemistry. Date herein show that 4H11 is relatively specific to high-grade ovarian serous carcinoma. Invasive lobular breast carcinoma is the major exception and shows extensive MUC16 protein as detected by 4H11. Lobular carcinoma of the breast has unique biology which is characterized by a propensity to metastasize to serosal surfaces (27). Since MUC16 is the cognate binding partner of mesothelin, this may have important implications for lobular cancer (28). The discordance rates for OC125 and 4H11 also suggest that 4H11 might provide additional, independent information from OC125 in a subset of ovarian carcinomas. Some tumors that are negative with OC125 retain cytoplasmic and extracellular portions of the MUC16 glycoprotein, portions of the molecule that are likely involved in transduction of signals potentially important in the malignant phenotype.

Thus, in one embodiment, the invention provides an isolated antibody, or an antigen-binding fragment thereof, that specifically binds to a polypeptide, or antigenic portion thereof, wherein the polypeptide is exemplified by a) MUC16 ectodomain polypeptide (exemplified by NFSPLAR RVDRVAIYEE FLRMTRNGTQ LQNFTLDRSS VLVDGYSPNR NEPLTGNSDL P (SEQ ID NO:17)), b) MUC16 cytoplasmic domain polypeptide (exemplified by VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18), which is contained within each of CGVLVTTRR RKKEGEYNVQ QQ (SEQ ID NO:03) and LVTTRR RKKEGEYNVQ QQ (SEQ ID NO:20)), and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19).

One advantage of the invention's antibodies is that the antibody internalizes into a cell, thereby being useful in applications for delivery inside a cell, such as disease therapy. "Internalized" when in reference to a molecule that is internalized by a cell refers to passage of the

molecule that is in contact with the extracellular surface of a cell membrane across the cell membrane to the intracellular surface of the cell membrane and/or into the cell cytoplasm. Methods for determining internalization are disclosed herein, including the detection of radiolabeled molecule inside the cell (Figure 5B).

In one embodiment, the invention's antibodies specifically bind to MUC16 ectodomain polypeptide that comprises a polypeptide selected from the group consisting of Polypeptide 1 NFSPLARRVDRVAIYEE (SEQ ID NO:01) and Polypeptide 2 TLDRSSVLVDGYSPNRNE (SEQ ID NO:02). Data herein show that the invention's antibodies specifically bind to GST-ΔMUC16^{c} ^{114} (Example 2, Table 1A). The specificity of the invention's antibodies is in contrast to prior art antibodies (e.g., VK8, M11 and OC125 antibodies) that did not bind to GST-ΔMUC16^{c114} purified protein or cell lysates of the SKOV3-phrGFP-ΔMUC16^{c114} cell line (Example 2, Figure 2).

In a further embodiment, the invention's antibodies lack specific binding to a glycosylated MUC16 extracellular domain, exemplified by the cleaved CA-125 described in Payne et al., U.S. Pat. No. 7,202,346.

While not intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:06 (i.e., the antibody 4H11 variable heavy (VH) chain amino acid sequence of Figure 8), and a variable light (V_L) chain encoded by SEQ ID NO:07 (i.e., the antibody 4H11 variable light (V_L) chain amino acid sequence of Figure 8). In a particular embodiment, the antibody is chimeric, wherein at least one of the V_L and V_H chains is fused to a human immunoglobulin constant region.

Also without intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to the Polypeptide 2 (SEQ ID NO:02) of the MUC16 ectodomain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:04 (i.e., the antibody 4A5 variable heavy (V_H) chain nucleotide sequence of Figure 8), and a variable light (V_L) chain encoded by SEQ ID NO:05 (i.e., the antibody 4A5 variable light (V_L) chain nucleotide sequence of Figure 8). In a particular embodiment, the antibody is chimeric wherein at least one of the V_L and V_H chains is covalently linked to a human immunoglobulin constant region.

Still without intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to the Polypeptide 1 (SEQ ID NO:01) of the MUC16 ectodomain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:08 (i.e., the antibody 9B11 variable heavy (VH) chain

nucleotide sequence of Figure 8), and a variable light (V_L) chain encoded by at least one of SEQ ID NO:09 (i.e., antibody 9B11 variable light (V_{LA}) chain nucleotide sequence of Figure 8), and SEQ ID NO:10 (i.e., the antibody 9B11 variable light (V_{LB}) chain nucleotide sequence of Figure 8). In a particular embodiment, the antibody is chimeric wherein at least one of the V_L and V_H chains is covalently linked to a human immunoglobulin constant region.

While not intending to restrict the source of antigen to which the invention's antibodies bind, in one embodiment, the MUC16 ectodomain polypeptide is expressed by a cell. Data herein show that the invention's exemplary antibodies bind to SKOV3 cells transduced with phrGFP-ΔMUC16^{c114} (Example 2).

While not limiting the sequence of antigen to which the invention's antibodies bind, in a further embodiment, the invention's antibodies specifically bind to a MUC16 cytoplasmic domain polypeptide that comprises VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18). In a particular embodiment, the MUC16 cytoplasmic domain polypeptide comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03). In some embodiment, the MUC16 cytoplasmic domain polypeptide is expressed by a cell. For example, data herein show that the invention's exemplary antibody binds to SKOV3 cells transduced with phrGFP-ΔMUC16^{c114} (Example 2). In a particular embodiment, the cell is permeabilized to facilitate internalization of the antibody into the cell so that it comes into contact with its cytoplasmic antigen.

Still without limiting the sequence of antigen to which the invention's antibodies bind, in a further embodiment, the invention's antibodies bind to a MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19). In a more preferred embodiment, the MUC16 extracellular domain polypeptide comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15).

Still without intending to limit the sequence of the V_L and V_H regions of the invention's antibodies, in one embodiment, the antibody specifically binds to Polypeptide 4 (SEQ ID NO:15) of the MUC16 extracellular domain polypeptide, wherein the antibody comprises a variable heavy (V_H) chain encoded by SEQ ID NO:11 (i.e., the antibody 24B3 variable heavy (V_H) chain amino acid sequence of Figure 8), and a variable light (V_L) chain encoded by SEQ ID NO:12 (i.e., the antibody 24B3 variable light (V_L) chain amino acid sequence of Figure 8).

The invention contemplates chimeric antibodies (see U.S. Pat. No. 7,662,387), monoclonal antibodies, recombinant antibodies, an antigen-binding fragment of a recombinant antibody, a humanized antibody, and an antibody displayed upon the surface of a phage (U.S. Pat. No. 7,202,346). In particular, the invention contemplates antibody fragments that contain the idiotype ("antigen-binding region" or "antigen-binding fragment") of the antibody molecule. For example,

such antigen-binding fragments include, but are not limited to, the Fab region, F(ab')2 fragment, pFc' fragment, and Fab' fragments.

The "Fab region" and "fragment, antigen binding region," interchangeably refer to portion of the antibody arms of the immnoglobulin "Y" that function in binding antigen. The Fab region is composed of one constant and one variable domain from each heavy and light chain of the antibody. Methods are known in the art for the construction of Fab expression libraries (Huse et al., Science, 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. In another embodiment, Fc and Fab fragments can be generated by using the enzyme papain to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment. The enzyme pepsin cleaves below the hinge region, so a "F(ab')2 fragment" and a "pFc' fragment" is formed. The F(ab')2 fragment can be split into two "Fab' fragments" by mild reduction.

The invention also contemplates a "single-chain antibody" fragment, i.e., an amino acid sequence having at least one of the variable or complementarity determining regions (CDRs) of the whole antibody, and lacking some or all of the constant domains of the antibody. These constant domains are not necessary for antigen binding, but constitute a major portion of the structure of whole antibodies. Single-chain antibody fragments are smaller than whole antibodies and may therefore have greater capillary permeability than whole antibodies, allowing single-chain antibody fragments to localize and bind to target antigen-binding sites more efficiently. Also, antibody fragments can be produced on a relatively large scale in prokaryotic cells, thus facilitating their production. Furthermore, the relatively small size of single-chain antibody fragments makes them less likely to provoke an immune response in a recipient than whole antibodies. Techniques for the production of single-chain antibodies are known (U.S. 4,946,778). The variable regions of the heavy and light chains can be fused together to form a "single-chain variable fragment" ("scFv fragment"), which is only half the size of the Fab fragment, yet retains the original specificity of the parent immunoglobulin.

The "Fc region" and "Fragment, crystallizable region" interchangeably refer to portion of the base of the immnoglobulin "Y" that function in role in modulating immune cell activity. The Fc region is composed of two heavy chains that contribute two or three constant domains depending on the class of the antibody. By binding to specific proteins, the Fc region ensures that each antibody generates an appropriate immune response for a given antigen. The Fc region also binds to various cell receptors, such as Fc receptors, and other immune molecules, such as complement proteins. By doing this, it mediates different physiological effects including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils. In an

experimental setting, Fc and Fab fragments can be generated in the laboratory by cleaving an immunoglobulin monomer with the enzyme papain into two Fab fragments and an Fc fragment.

The invention contemplates polyclonal antibodies and monoclonal antibodies. "Polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Generic methods are available for making polyclonal and monoclonal antibodies that are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to hamsters, rabbits, mice, rats, sheep, goats, *etc.* For preparation of monoclonal antibodies, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (See e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include, but are not limited to, the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature, 256:495-497 (1975)), techniques using germ-free animals and utilizing technology such as that described in PCT/US90/02545, as well as the trioma technique, the human B-cell hybridoma technique (See e.g., Kozbor et al., Immunol. Today, 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 (1985)). In some particularly preferred embodiments of the present invention, the present invention provides monoclonal antibodies.

Also contemplated are chimeric antibodies. As used herein, the term "chimeric antibody" contains portions of two different antibodies, typically of two different species. See, *e.g.*: U.S. Pat. No. 4,816,567 to Cabilly et al.; U.S. Pat. No. 4,978,745 to Shoemaker et al.; U.S. Pat. No. 4,975,369 to Beavers et al.; and U.S. Pat. No. 4,816,397 to Boss et al. Chimeric antibodies include monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer (H_2L_2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a Hc region that aggregates (e.g., IgM H chain).

The invention also contemplates "humanized antibodies," i.e., chimeric antibodies that have constant regions derived substantially or exclusively from human antibody constant regions, and variable regions derived substantially or exclusively from the sequence of the variable region from a mammal other than a human. Humanized antibodies preferably have constant regions and variable regions other than the complement determining regions (CDRs) derived substantially or

exclusively from the corresponding human antibody regions and CDRs derived substantially or exclusively from a mammal other than a human. Thus, in one embodiment, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are generally made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a nonhuman immunoglobulin and all or substantially all of the FR residues are those of a human immunoglobulin sequence. The humanized antibody may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Humanized antibodies may be generated using methods known in the art, e.g., U.S. Pat. No. 5,225,539 to Winter et al., including using human hybridomas (Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A.80:2026-2030 (1983)) or by transforming human B cells with EBV virus in vitro (Cole *et al.*, *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 (1985)). Additional methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes (U.S. Pat. Nos. 5,545,806; 5,569,825 and 5,625,126). Humanized antibodies may also be made by substituting the complementarity determining regions of, for example, a mouse antibody, into a human framework domain (PCT Pub. No. WO92/22653).

Importantly, early methods for humanizing antibodies often resulted in antibodies with lower affinity than the non-human antibody starting material. More recent approaches to humanizing antibodies address this problem by making changes to the CDRs. See U.S. Patent Application Publication No. 20040162413. In some embodiments, the invention's humanized antibodies contain an optimized heteromeric variable region (e.g. that may or may not be part of a full antibody other molecule) having equal or higher antigen binding affinity than a donor heteromeric variable region, wherein the donor heteromeric variable region comprises three light chain donor CDRs, and wherein the optimized heteromeric variable region comprises: a) a light chain altered variable region comprising; i) four unvaried human germline light chain framework regions, and ii) three light chain altered variable region

CDRs, wherein at least one of the three light chain altered variable region CDRs is a light chain donor CDR variant, and wherein the light chain donor CDR variant comprises a different amino acid at only one, two, three or four positions compared to one of the three light chain donor CDRs (e.g. the at least one light chain donor CDR variant is identical to one of the light chain donor CDRs except for one, two, three or four amino acid differences).

Chimeric antibodies containing amino acid sequences that are fused to constant regions from human antibodies, or to toxins or to molecules with cytotoxic effect, are known in the art (e.g., U.S. Pat. Nos. 7,585,952; 7,227,002; 7,632,925; 7,501,123; 7,202,346; 6,333,410; 5,475,092; 5,585,499; 5,846,545; 7,202,346; 6,340,701; 6,372,738; 7,202,346; 5,846,545; 5,585,499; 5,475,092; 7,202,346; 7,662,387; 6,429,295; 7,666,425; and 5,057,313).

Antibodies that are specific for a particular antigen may be screened using methods known in the art (e.g., U.S. Pat. No. 7,202,346) and disclosed herein. For example, In the production of antibodies, screening for the desired antibody can be accomplished by radioimmunoassay, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or radioisotope labels), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*

In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. As is well known in the art, the immunogenic peptide should be provided free of the carrier molecule used in any immunization protocol. For example, if the peptide was conjugated to KLH, it may be conjugated to BSA, or used directly, in a screening assay.

In one embodiment, the invention's antibodies are monoclonal antibodies produced by a hybridoma cell line. In a particular embodiment, the monoclonal antibody specifically binds to a MUC16 ectodomain polypeptide that comprises Polypeptide 1 (SEQ ID NO:01), as exemplified by the antibody selected from the group consisting of 9B11.20.16, 10A2, 2F4, 23D3, 30B1, and 31B2 (Tables 1 and 2). In a preferred embodiment, the antibody is 9B11.

In another embodiment, the monoclonal antibody specifically binds to a MUC16 ectodomain polypeptide that comprises Polypeptide 2 (SEQ ID NO:02), wherein the antibody is exemplified by 4H11.2.5 , 13H1, 29G9, 9C9.21.5.13, 28F8, 23G12, 9C7.6, 11B6, 25G4, 5C2.17,

4C7, 26B2, 4A5.37, 4A2, 25H3, and 28F7.18.10 (Tables 1 and 2). In a preferred embodiment, the antibody is exemplified by 4H11.2.5, 4A5.37, 9C9.21.5.13, 28F7.18.10, 9C7.6, and 5C2.17.

In a further embodiment, the monoclonal antibody specifically binds to a MUC16 cytoplasmic domain polypeptide that comprises Polypeptide 3 CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03), wherein the antibody is exemplified by 31A3.5.1, 19D1, 10F6, 22E10, 22F1, 3H8, 22F11, 4D7, 24G12, 19G4, 9A5, 4C2, 31C8, 27G4, and 6H2 (Tables 1 and 2). In a preferred embodiment, the antibody is 31A3.5.1.

In another embodiment, the monoclonal antibody specifically binds to a MUC16 extracellular domain polypeptide that comprises Polypeptide 4 KSYF SDCQVSTFRS VPNRHHTGVD SLCNFSPL (SEQ ID NO:15), wherein the antibody is exemplified by 24B3 and 9C7 (Table 2).

The invention's antibodies and methods for their use (both diagnostic and therapeutic) are disease specific. "Specificity" of a method and/or molecule for disease, such as "specificity for cancer" which is interchangeably used with "cancer specificity", refers to the proportion (e.g., percentage, fraction, etc.) of negatives (i.e., healthy individuals not having disease) that are correctly identified, i.e., the percentage of healthy subjects who are correctly identified as not having disease. Specificity may be calculated according to the following equation:

Specificity = number of true negatives / (number of true negatives + number of false positives).

Thus, in some embodiments, the invention's compositions and/or methods have a "cancer specificity" greater than 50%, including any numerical value from 51% to 100%, such as 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 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%, and 99%. While a 100% specificity is most desirable, i.e., not predicting anyone from the healthy group as having cancer, it is not necessary. Data herein demonstrate the invention's cancer specificity (Table 3).

In alternative embodiments, specificity is expressed (together with sensitivity) as a statistical measure of the performance of a binary classification test, such as using a Receiver Operator Characteristic (ROC) curve". For any test, there is usually a trade-off between specificity and sensitivity. For example: in cancer screening tests of human subjects, it is undesirable to risk falsely identifying healthy people as having cancer (low specificity), due to the high costs. These costs are both physical (unnecessary risky procedures) and financial. This trade-off can be represented graphically using a ROC curve. "Receiver Operator Characteristic curve" and "ROC

curve" refer to a plot of the true positive rate (AKA sensitivity) versus true negative rate (AKA 1-specificity). The measured result of the test is represented on the x axis while the y axis represents the number of control (e.g., healthy) or case (e.g., cancer) subjects. For any given cut point (each point along the x axis) a sensitivity and specificity of the assay can be measured. The range of sensitivity and specificity for any given assay can range from 0% to 100%, depending on the selected cut point. For this reason, in some preferred embodiments, the AUC is used as the standard measure of an assay's specificity and/or sensitivity. The "area under the curve" ("AUC") for the ROC curve plot is equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. Thus, AUC is a general measure of a tests ability to successfully discriminate between case (e.g., cancer) and control (e.g., healthy) subjects. Random chance would generate an AUC of 0.5. Therefore, in one embodiment, useful tests preferably have AUC's greater than 0.50, including any value from 0.51 to 1.00, such as from 0.55 to 1.00, from 0.60 to 1.00, from 0.65 to 1.00, from 0.70 to 1.00, from 0.75 to 1.00, from 0.80 to 1.00, from 0.85 to 1.00, from 0.90 to 1.00, from 0.95 to 1.00, and most preferably 1.00. AUC values greater than 0.50 include 0.51, 0.52, 0.52, 0.54, 0.55, 0.56, 0.57, 0.58, 0.59, 0.60, 0.61, 0.62, 0.63, 0.64, 0. 65, 0.66, 0.67, 0. 68, 0.69, 0.70, 0.71, 0.72, 0.73, 0.74, 0.75, 0.76, 0.77, 0.78, 0.79, 0.80, 0.81, 0.82, 0.83, 0.84, 0.85, 0.86, 0.87, 0.88, 0.89, 0.90, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, and 0. 99.

The invention's antibodies and methods for their use (both diagnostic and therapeutic) are disease sensitive. "Sensitivity" of a method and/or molecule for disease, such as "sensitivity for cancer" which is interchangeably used with "cancer sensitivity," refers to the proportion (e.g., percentage, fraction, etc.) of positives (i.e., individuals having cancer) that are correctly identified as such (e.g. the percentage of people with cancer who are identified as having the condition). Sensitivity may be calculated according to the following equation; Sensitivity = number of true positives / (number of true positives + number of false negatives).

Thus, in some embodiments, the invention's compositions and/or methods have a "disease sensitivity," such as "cancer sensitivity," greater than 50%, including any numerical value from 51% to 100%, such as 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 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%, and 99%. While a 100% sensitivity is most desirable (i.e., predicting all subjects from the cancer group as having cancer), it is not necessary.

In alternative embodiments, the invention's compositions and/or methods have a "disease sensitivity," such as "cancer sensitivity," equal to or lower than 50%, including any numerical

value from 0% to 50%, such as 1%, 2%, 3%, 4%, 6%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, and 49%.

In some embodiments, sensitivity is expressed (together with specificity) as a statistical measure of the performance of a binary classification test, such as using AUC of a ROC curve, as discussed above with respect to specificity.

D. Hybridoma Cell Lines

In addition to the invention's novel antibodies, the invention also provides hybridoma cell lines that produce these antibodies. "Hybridoma cell" refers to a cell line produced by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis. The antibodies produced by the hybridoma cell are all of a single specificity and are therefore monoclonal antibodies (in contrast to polyclonal antibodies).

In a particular embodiment, the invention provides hybridoma cell lines that produce a monoclonal antibody that specifically binds to a polypeptide, or antigenic portion thereof, selected from the group consisting of a) MUC16 ectodomain polypeptide (e.g., NFSPLAR RVDRVAIYEE FLRMTRNGTQ LQNFTLDRSS VLVDGYSPNR NEPLTGNSDL P (SEQ ID NO:17)), b) MUC16 cytoplasmic domain polypeptide (e.g., VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18)), and c) MUC16 extracellular domain polypeptide that contains a cysteine loop polypeptide CQVSTFRSVPNRHHTGVDSLC (SEQ ID NO:19). The MUC16 polypeptide SEQ ID NO:18 is contained within LVTTRR RKKEGEYNVQ QQ (SEQ ID NO:20). Thus, SEQ ID NO:20 contains both a transmembrane domain amino acid (L) and a cytoplasmic domain portion VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18), i.e., the L is optional, as it is part of the transmembrane domain. The MUC16 polypeptide SEQ ID NO:18 is also contained within CGVLVTTRR RKKEGEYNVQ QQ (SEQ ID NO:03). Thus, SEQ ID NO:03 contains both a transmembrane domain portion (CGVL) and a cytoplasmic domain portion VTTRR RKKEGEYNVQ QQ (SEQ ID NO:18), i.e., the CGVL is optional, as it is part of the transmembrane domain.

E. Conjugates Of The Invention's Antibodies Linked To Cytotoxic Agents And/Or Prodrugs

The invention contemplates conjugate antibodies. A "conjugate" antibody refers to an antibody of the present invention covalently linked to a cytotoxic agent and/or a prodrug of a cytotoxic agent.

"Cytotoxic agent" refers any agent that is capable of reducing the growth of, and/or killing, a target cell. A "prodrug" represents an analog of a cytotoxic agent that substantially lacks cytotoxic activity until subjected to an activation step. Activation steps may include enzymatic cleavage, a chemical activation step such as exposure to a reductant, or a physical activation step such as photolysis.

The covalent linkage between the invention's antibodies and the cytotoxic agent or prodrug can include cleavable linkages such as disulfide bonds, which may advantageously result in cleavage of the covalent linkage within the reducing environment of the target cell. Such conjugates are useful as tumor-cell specific therapeutic agents.

In one embodiment, the cytotoxic agent is a small drug molecule (Payne et al., U.S. Pat. No. 7,202,346). In another embodiment, the cytotoxic agent a maytansinoid, an analog of a maytansinoid, a prodrug of a maytansinoid, or a prodrug of an analog of a maytansinoid (U.S. Pat. Nos. 6,333,410; 5,475,092; 5,585,499; 5,846,545; 7,202,346). In another embodiment, the cytotoxic agent may be a taxane (see U.S. Pat. Nos. 6,340,701 & 6,372,738 & 7,202,346) or CC-1065 analog (see U.S. Pat. Nos. 5,846,545; 5,585,499; 5,475,092 & 7,202,346).

In another embodiment, the cytotoxic agent is exemplified by an auristatin, a DNA minor groove binding agent, a DNA minor groove alkylating agent, an enediyne, a duocarmycin, a maytansinoid, and a vinca alkaloid (U.S. Pat. No. 7,662,387).

In a further embodiment, the cytotoxic agent is an anti-tubulin agent (U.S. Pat. No. 7,662,387). In yet another embodiment, the cytotoxic agent is exemplified by dimethylvaline-valine-dolaisoleuine-dolaproine-phenylalanine-p-phenylenediamine (AFP), dovaline-valine-dolaisoleunine-dolaproine-phenylalanine (MMAF), and monomethyl auristatin E (MAE) (U.S. Pat. No. 7,662,387).

In an additional embodiment the toxic agent is exemplified by radioisotope emitting radiation, immunomodulator, lectin, and toxin (U.S. Pat. No. 6,429,295). In particular, the radioisotope emitting radiation is an alpha-emitter selected from the group consisting of ^{212}Bi, ^{213}Bi, and ^{211}At, or a beta-emitter selected from the group consisting of ^{186}Re and ^{90}Y, or a gamma-emitter ^{131}I (U.S. Pat. No. 7,666,425).

In an alternative embodiment, the toxin is exemplified by ricin, the A-chain of ricin, and pokeweed antiviral protein (U.S. Pat. No. 5,057,313).

In yet another embodiment, the cytotoxic agent is an anti-cancer drug selected from the group consisting of methotrexate, 5-fluorouracil, cycloheximide, daunomycin, doxorubicin, chlorambucil, trenimon, phenylenediamine mustard, adriamycin, bleomycin, cytosine arabinoside or Cyclophosphamide (U.S. Pat. No. 5,057,13).

F. Detecting Muc16 Portions And Diagnostic Applications

The invention provides a method for detecting a disease that comprises overexpression of MUC16 in a subject, wherein the method comprises a) providing i) a sample from a subject, and ii) any one or more of the invention's antibodies, b) contacting the sample with the antibody under conditions for specific binding of the antibody with its cognate antigen, and c) detecting an increased level of binding of the antibody to the sample compared to a control sample lacking the disease, thereby detecting the disease in the subject. Generic methods for detecting disease using antibodies are known in the art (Payne et al., U.S. Pat. No. 7,202,346). The invention's methods are particularly useful in detecting cancer, such as ovarian cancer and breast cancer.

The invention's methods are not limited to a particular approach to detecting binding of the invention's antibodies to their antigens. In one embodiment, detecting binding to the invention's antibodies typically involves using antibodies that are labeled with a detectable moiety, such as radioisotope (e.g., ^{3}H, ^{14}C, ^{32}P, ^{35}S, and/or ^{125}I), fluorescent or chemiluminescent compound (e.g., fluorescein isothiocyanate, rhodamine, and/or luciferin) and/or an enzyme (e.g., alkaline phosphatase, beta-galactosidase and/or horseradish peroxidase).

Methods for conjugating antibodies to a detectable moiety are known in the art (e.g., Hunter, et al., Nature 144:945 (1962); David, e at., Biochemistry 13:1014 (1974); Pain, et al., J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

Thus, the invention's antibodies may be employed in immunoassays, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays, including immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), and Western blots.

For example, with respect to immunohistochemical detection, data herein demonstrate that antibody 4H11 is useful in detecting high-grade ovarian serous carcinoma, lobular cancer (28), and a subset of ovarian carcinomas that are negative with OC125 and that retain cytoplasmic and extracellular portions of the MUC16 glycoprotein.

The antibodies of the invention also are useful for radiographic *in vivo* imaging, wherein an antibody labeled with a detectable moiety such as a radio-opaque agent or radioisotope is administered to a subject, preferably into the bloodstream, and the presence and location of the labeled antibody in the host is assayed. This imaging technique is useful in the staging and treatment of malignancies.

The invention's antibodies are additionally useful as affinity purification agents. In this process, the antibodies are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art, to capture and purify molecules that contain antigens that specifically bind to the invention's antibodies.

G. Therapeutic Applications

The invention provides methods for treating a disease that comprises overexpression of MUC16, comprising administering to a subject having the disease a therapeutically effective amount of any one or more of the invention's antibodies. Generic methods for treating disease with antibodies are known in the art (Payne et al., U.S. Pat. No. 7,202,346). The invention's methods are particularly useful in treating cancer, such as ovarian cancer and breast cancer. These methods are also applicable to primary cancer, metastatic cancer, and recurrent cancer.

The term "administering" to a subject means providing a molecule to a subject. This may be done using methods known in the art (*e.g.*, Erickson et al., U.S. Patent 6,632,979; Furuta et al., U.S. Patent 6,905,839; Jackobsen et al., U.S. Patent 6,238,878; Simon et al., U.S. Patent 5,851,789). The invention's compositions may be administered prophylactically (*i.e.*, before the observation of disease symptoms) and/or therapeutically (*i.e.*, after the observation of disease symptoms). Administration also may be concomitant with (*i.e.*, at the same time as, or during) manifestation of one or more disease symptoms. Also, the invention's compositions may be administered before, concomitantly with, and/or after administration of another type of drug or therapeutic procedure (*e.g.*, surgery). Methods of administering the invention's compositions include, without limitation, administration in parenteral, oral, intraperitoneal, intranasal, topical and sublingual forms. Parenteral routes of administration include, for example, subcutaneous, intravenous, intramuscular, intrasternal injection, and infusion routes.

In one embodiment, the invention's compositions comprise a lipid for delivery as liposomes. Methods for generating such compositions are known in the art (Borghouts et al. (2005). J Pept Sci *11*, 713-726; Chang et al. (2009) PLoS One *4*, e4171; Faisal et al. (2009) Vaccine *27*, 6537-6545; Huwyleret al. (2008) Int J Nanomedicine 3, 21-29; Song et al. (2008) Int J Pharm 363, 155-161; Voinea et al. J Cell Mol Med 6, 465-474).

Antibody treatment of human beings with cancer is known in the art, for example in U.S. Pat. Nos. 5,736,137; 6,333,410; 5,475,092; 5,585,499; 5,846,545; 7,202,346; 6,340,701; 6,372,738; 7,202,346; 5,846,545; 5,585,499; 5,475,092; 7,202,346; 7,662,387; 7,662,387; 6,429,295; 7,666,425; 5,057,313.

The invention's antibodies may be administered with pharmaceutically acceptable carriers, diluents, and/or excipients. Examples of suitable carriers, diluents and/or excipients include: (1) Dulbecco's phosphate buffered saline, pH about 7.4, containing about 1 mg/ml to 25 mg/ml human serum albumin, (2) 0.9% saline (0.9% w/v NaCl), and (3) 5% (w/v) dextrose.

The invention's antibodies are typically administered in a therapeutic amount. The terms "therapeutic amount," "pharmaceutically effective amount," "therapeutically effective amount," and "biologically effective amount," are used interchangeably herein to refer to an amount that is sufficient to achieve a desired result, whether quantitative or qualitative. In particular, a pharmaceutically effective amount is that amount that results in the reduction, delay, and/or elimination of undesirable effects (such as pathological, clinical, biochemical and the like) that are associated with disease. For example, a "therapeutic amount that reduces cancer" is an amount that reduces, delays, and/or eliminates one or more symptoms of cancer.

For example, specific "dosages" of a ""therapeutic amount" will depend on the route of administration, the type of subject being treated, and the physical characteristics of the specific subject under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical, veterinary, and other related arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors, which those skilled in the art will recognize. The dosage amount and frequency are selected to create an effective level of the compound without substantially harmful effects.

When present in an aqueous dosage form, rather than being lyophilized, the antibody typically will be formulated at a concentration of about 0.1 mg/ml to 100 mg/ml.

Depending on the type and severity of the disease, about 0.015 to 15 mg of antibody/kg of patient weight is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs.

The methods of the present invention can be practiced *in vitro*, *in vivo*, or *ex vivo*.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Materials And Methods

The following is a brief description of the exemplary materials and methods used in the subsequent Examples.

Cell Cultures:

OVCAR3, SKOV3, and A2780 cell lines were obtained through the American Type Culture Collection (ATCC, Manassas, VA) and sustained in culture according to the ATCC literature. For the creation of MUC16+ transfected cell lines, the carboxyterminus portion of the MUC16 cDNA was introduced as green fluorescent protein fusion proteins using the Vitality® phrGFP vector expression system (Stratagene, La Jolla, CA). Stable cell lines were selected using geneticin (G418, Invitrogen, Grand Island, NY) in their respective culture media and isolated by expression of Green Fluorescence Protein. Stable transfectants were routinely maintained in G418 in their culture media respectively. The $\Delta MUC16^{c114}$ transfectants have cell surface expression of MUC16 protein from the putative cleavage site to the carboxyterminus (AA 1776 to 1890) (12).

Monoclonal Preparation:

Using the MUC16 sequence, peptide sequences encoding elements of the $\Delta MUC16^{c114}$ amino acid sequence were synthesized at the Memorial Sloan-Kettering Cancer Center (MSKCC) Microchemistry Core Facility. The inventors synthesized 3 polypeptides (Figure 1) and modified Polypeptide 1 and Polypeptide 2 with a cysteine at the N-terminus for better conjugation to KLH. Equal concentrations of the KLH-conjugated peptides were mixed and then used as the immunogen for 5 BALB/c mice. The inventors selected 1 of the 5 mice whose serum showed the highest reactivity to individual peptides by ELISA, and the MSKCC Monoclonal Antibody Core Facility performed the fusion and selected the antibodies using standard protocols. After 10 days of fusion, supernatants were selected and screened for reactivity by ELISA against the individual synthetic peptides.

ELISA:

Sandwich ELISA was performed to see the positivity of the antibodies to individual peptides and GST-$\Delta MUC16^{c114}$ fusion protein following routine core facility protocol for ELISA assay.

FACS Analyses:

Adherent target cells were removed by 0.05% Trypsin and 0.1% EDTA, washed, and counted by a hemocytometer. Cells were distributed into multiple Eppendorf tubes with at least 0.5-1 x 10^6 cells per tube. Cells were washed with phosphate buffered saline (PBS) containing 1% FCS and 0.025% Sodium Azide (FACS buffer). For internal FACS staining, cells in the Eppendorf tubes were permeabilized with 1:10 diluted FACS Permeabilizing Solution 2 (BD BioSciences, San Jose, CA) for 10 minutes at room temperature and then washed twice with ice cold FACS buffer. Then they were incubated either without (for second antibody control) or with 1 µg/tube of bioreactive supernatants of mouse MUC16 monoclonals for 30 minutes on ice. For surface FACS staining, cells were incubated either without (for second antibody control) or with 1 µg/tube of bioreactive supernatants of MUC16 monoclonals (9B11.20.16, 9C9.21.5.13 and 4H11.2.5), Mouse anti-human OC125 (M3519), Mouse anti-human M11 (M3520) (DakoCytomation, Dako North America Inc., Carpinteria, CA) or VK8 (kindly provided by Dr. Beatrice Yin and Dr. Ken Lloyd, MSKCC, New York, NY) for 30 minutes on ice. Cells in Eppendorf tubes were also surface stained with 1 µg/tube of non-specific isotype matched control mouse antibodies (13C4 for IgG1 and 4E11 for IgG2b monoclonals obtained from MSKCC Monoclonal Core Facility) and incubated on ice for 30 minutes. All cells were washed three times with FACS buffer. Cells were incubated with 1 µg/tube of second antibody Goat anti-mouse IgG1-PE or IgG2b-PE for 30 minutes on ice and then washed three times with FACS buffer. The cells were analyzed by a FACScalibur™ machine at the MSKCC Flow Cytometry Core Facility.

Western Blot Analysis:

Stable cell lines were cultured in 10 cm dishes in their respective culture media and incubated with 5% CO_2 at 37°C for 3 days. They were washed twice with ice cold PBS to remove the serum-containing media. Adherent cells were scraped with 1-2 ml of ice cold PBS, and the cells were spun down in an Eppendorf tube at 4°C in an Eppendorf centrifuge. Supernatant was discarded, and the cells were lysed with 0.2 ml of modified Ripa lysis buffer (20 mM Tris-HCL; pH 7.4; 150 mM NaCl; 1% NP-40; 1 mM Na3VO4; 1 mM PMSF; 1 mM DTT; 10 µg/ml leupeptin; and 10 µg/ml aprotinin) for 30 minutes on ice and spun at 4°C for 10 minutes. The soluble solution was separated into a tube and the debris pellet was discarded. Protein concentration was measured using the Bio-Rad Protein Assay (BioRaD Laboratories, Hercules, CA). Equal amounts of proteins (GST-MUC16-CD-fusion protein or stable cell line extracts) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane using a BioRad transfer apparatus in a cold room at 4°C. The membranes were blocked with 3% bovine serum albumin (BSA) in PBS with 0.1% TWEEN® 20 (PBST) at 4°C overnight. Membranes were probed with primary antibody (1:1000

dilution) for 1 hr at room temperature and then washed three times with PBST. Then the membranes were stained with corresponding second antibody, anti-Mouse IgG Horse Radish Peroxidase (HRP) linked whole antibody from sheep (GE Healthcare, UK) (1:5000 dilution), for 1 hr at room temperature. Membranes were washed three times with PBST and developed with a Western Lightning® chemiluminescence reagent (ECL, Perkin Elmer, Waltham, MA) for 1-5 minutes at room temperature, and the signals were developed on Kodak® BioMax® Film.

Binding and internalization studies with monoclonal antibodies and OVCAR3 and SKOV3 stable transfectants:

Purified monoclonal antibodies were labeled with ^{131}I using the iodogen method and purified by size exclusion chromatography (22). Saturation binding studies were performed with radiolabeled antibodies using substrates of intact OVCAR-3 cells. Briefly, 10 test solutions were prepared (in triplicate) and they contained increasing amounts of the radioiodinated antibodies, 3-500 000 cells in a total volume of 500 µL of PBS (0.2 % BSA; pH 7.4). The cells were isolated by rapid filtration through a glass fiber membrane and washed with ice cold tris buffered saline. Cells were counted in a gamma counter with standards of total activity added. For each concentration of radiolabeled antibody, non-specific binding was determined in the presence of 100 nM of the unmodified antibody. The data were analyzed with a least squares regression method (Origin, Microcal, Software Inc., Northampton, MA) to determine the K_d and B_{max} values, and a Scatchard transformation was performed.

Antibody cell internalization studies were performed with ^{131}I-4H11 and ^{131}I-OC125 monoclonal antibodies and SKOV3-phrGFP-ΔMUC16^{c334} stable transfected cells. Briefly, radiolabeled antibody (370 MBq/mg, 100 kcpm) in 2 mL of medium was added to SKOV3 cells plated in a 6-well plate. The plates were incubated at 37°C for up to 24 hours. At various time points, the medium was removed from three wells and the cells washed with 2 x 2 mL PBS. Cell surface bound activity was then stripped and collected with 2 x 2 mL of an ice cold acid wash (100 mM acetic acid 100 mM glycine; pH 3.0). The cells were then dissolved with 2 x 1 ml 1 M NaOH and collected. At the end of the study all samples were counted with a gamma counter together with standards, representing the initial amount of radioactivity added. All the media samples were analyzed by ITLC-SG with mobile phases of 5% TCA to determine unbound ^{131}I.

Tissue microarray (TMA):

Tissue microarrays were either constructed within our institution or bought from a commercial laboratory if not available internally. Briefly, core-needle biopsies of pre-existing paraffin-embedded tissue were obtained from the so-called donor blocks and then relocated into a recipient paraffin-arrayed "master" block by using the techniques by Kononen et al. and subsequently modified by Hedvat et al

(23-24). A manually operated Tissue Arrayer MTA-1 from Beecher Instruments Inc. (Sun Prairie, WI) was used to produce sample circular spots (cores) that measured 0.6 to 1.0 mm in diameter. The cores were arrayed 0.3 to 0.4 mm apart from each other. A layer of control tissues was strategically laid around the actual tissue microarrays in order to avoid edging effects. The specific composition of each tissue microarray is delineated below. Slides of tissue microarrays for ovarian cancer, prostate cancer, adenocarcinoma of the lung, mucinous neoplasms of the pancreas, and invasive ductal and invasive lobular breast carcinoma were prepared by cutting 4 um sections from formalin-fixed paraffin-embedded tissue. Normal adult and fetal tissue microarrays were obtained from a commercial source (Biomax, US). OVCAR3 cells were used as positive controls.

Immunohistochemistry:

Immunohistochemistry was performed on the tissue microarrays with both standard OC125 (Ventana, Tuscon, AZ) and the novel monoclonal antibodies. Sections of the tissue microarrays were cut at 4 microns, placed on Superfrost™/Plus microscope slides (Fisher brand) and baked in a 60° oven for at least 60 minutes. The slides were then deparaffinized and hydrated to distilled water, soaked in citrate buffer at pH 6.00 for 30 minutes at 97° C, washed in running water for 2-5 minutes, incubated for 5 minutes in 3% hydrogen peroxide diluted in distilled water. Slides were washed in distilled water for 1 minute, transferred to a bath of phosphate buffered saline (PBS), pH 7.2, for two changes of 5 minutes each and placed in 0.05% BSA diluted in PBS for a minimum of 1 minute. After drying around tissue sections, normal serum was applied at a 1:20 dilution in 2% BSA/PBS and incubated for a minimum of 10 minutes at room temperature in a humidity chamber. The serum was then suctioned off without allowing the sections to dry, and approximately 150 lambda of novel antibody at a dilution of 1:1000 was placed on the tissue. The slide was incubated overnight (approximately 15-18 hours) at 4° C in a humidity chamber. Primary antibody was washed off using three changes of PBS for 10 minutes each. Secondary antibody, biotinylated α-

mouse from Vector laboratories (Burlingame, Ca), was applied at 1:500 dilution in 1% BSA/PBS and incubated for 45-60 minutes at room temperature in humidity chamber. The antibody was washed off again using three changes of PBS as above. Slides were then transferred to a bath of diaminobenzidine (DAB), diluted in PBS for 5-15 minutes. The slides were then washed in tap water for 1 minute, counterstained using Harris modified hematoxylin (Fisher), decolorized with 1% acid alcohol and blue in ammonia water, dehydrated with 3 changes each of 95% ethanol, 100% ethanol and xylene for 2 minutes each and coverslipped with permanent mounting medium.

Immunohistochemistry scoring:

Commercially available antibodies, such as OC125 and M11, target complex glycosylation-dependent epitopes. Our hypothesis is that glycosylation may be tissue specific; therefore, it was important to examine the utility of the peptide-directed antibodies in paraffin-fixed tissues and survey the prevalence of MUC16 expression. The three candidate antibodies, 4H11, 9C9 and 4A5, were characterized using OVCAR3 cell line pellets. Of the three, the 4H11 antibody showed the strongest, most diffuse and consistent staining pattern at multiple dilutions, with the least amount of background staining and, therefore, was optimized for use in human tissues in the pathology core facility.

Using 4H11, the inventors stained and scored positivity using tissue microarrays from high-stage, high-grade ovarian serous carcinomas (Figure 2), these tumors being the most common type of ovarian cancer, representing approximately 80-85% of all ovarian carcinomas in Western industrialized nations (25). To test the specificity of the novel antibody, the inventors also stained tissue microarrays of cancers of the prostate, lung, breast, and pancreas and compared their staining intensities with that of OC125 monoclonal antibody (Figure 6A-D). To determine whether there would be any cross-reactivity with normal human tissues, the antibodies were also tested on normal human adult and fetal TMAs.

All of the stained sections were reviewed by a reference pathologist (KJP). A subset of cores for which there was equivocal staining was also independently scored by a second pathologist (RAS) to ensure consistency in scoring methods. Only cytoplasmic and/or membranous staining was considered positive. If a portion of the cell showed membranous staining, that was considered partial staining. A scoring system was devised to provide a semiquantitative assessment of staining distribution and intensity in individual cores. At the same time, it was designed to be useful for comparing the staining distribution and intensity between OC125 and the novel antibodies. The score incorporated the percentage of cells, the intensity and pattern of the staining according to the following standards: score 0: no staining; score 1: <5% strong or weak; score 2: 5-50% strong or

weak; score 3: 51-75% strong or 51-100% weak; score 4: 76-99% strong; and score 5: 100% strong staining (Figure 3). The pathologist first reviewed all tissue microarrays stained with OC125 and scored each core. Then the same cores stained with the novel antibodies were scored 1 to several days after OC125 without reference to the previous results. Direct comparison of the scoring between the stains for each core was made only after all of the scoring was completed. The same process was used for all non-ovarian tissue microarrays. After comparison, core staining was determined to be concordant, equivocal, or discordant based on the point differentials. Concordant cores differed by 0 to 1 point, equivocal cores differed by 2 points, and discordant cores differed by 3 to 5 points. The one exception to this rule was when the difference of 1 point was between a score of 0 and 1, in which case, the differences were considered equivocal. This was in order to truly separate negative cases from even focally positive ones.

EXAMPLE 2

Generation and characterization of anti-MUC16 monoclonal antibodies

MUC16-directed monoclonal antibodies were isolated by ELISA-based screening using both the individual peptides and recombinant GST-ΔMUC16^{c114} protein followed by sequential subcloning for single cell clones.

Tables 1A and 1B: MUC16-carboxyterminus monoclonal antibodies showing their reactivity to GST-ΔMUC16^{c114} western, FACS analysis on OVCAR3 wild type cells

Table 1A

	Peptide 1				Peptide 2				Peptide 3			
ELISA Hybridoma Sups (1:1)	(1:10) GST-MucCD Western +/-	(1:1) OVCAR3 FACS +/-	Isotype	ELISA Hybridoma Sups (1:1)	(1:10) GST-MucCD Western +/-	(1:1) OVCAR3 FACS +/-	Isotype	ELISA Hybridoma Sups (1:1)	(1:10) GST-MucCD Western +/-	(1:1) OVCAR3 FACS +/-	Isotype
10A2	+	-	IgG1,IgM	13H1	Weak	-	IgG1	22E10	+	-	IgG2b
23D4	-	-	missing	28F8	+	+	IgG1,IgM	22F11	Weak	-	IgM
2F4	Weak	-	IgG1,IgM	11B6	-	-	IgM	19G4	Weak	-	IgG1,IgM
9B11	Weak	+/-	IgG1	4C7	+	-	IgG1	31A3	Weak	-	IgG1
23D3	Weak	+	IgG1,IgG2b	28F7	+	+	IgG1	4C2	+	-	IgG1,IgM
30B1	-	-	IgG1	9C7	+	+	IgG1	27G4	+	-	IgM
31B2	+	-	IgM	9C9	+	+	IgG1, IgG2b	19D1	+	-	IgG2b
				4H11	+	+	IgG2b, IgM	22F1	+	-	IgG2b,IgM
				4A2	-	-	IgG1	4D7	+	-	IgG3
				4A5	+	+	IgG1	9A5	-	-	IgM
				29G9	+	-	IgG1	31C8	-	-	IgG2b
				5C2	+	+	IgG1	6H2	Weak	-	IgG1,IgM
				23G12	-	-	IgG1,IgG2a	10F6	-	-	IgG1
				25G4	-	-	IgG1,IgM	3H8	+	-	IgG1,IgM
				26B2	+	+	IgG1,IgG2b,IgM	24G12	-	-	IgG1,IgM
				25H3	-	-	IgG1,IgM				

Table 1B

	Peptide 1				Peptide 2				Peptide 3		
		OVCAR3 FACS +/-	Isotype			OVCAR3 FACS +/-	Isotype			OVCAR3 FACS +/-	Isotype
9B11.20.16		+/-	IgG1	9C9.21.5.13		+	IgG2b	31A3.5.1		-	IgG1
				4H11.2.5		+	IgG2b				
				9C7.6		+	IgG1				
				5C2.17		+	IgG1				
				4A5.37		+	IgG1				
				28F7.18.10		+	IgG1				

Table 2: Antibodies specific for exemplary portions of MUC16

1. Muc16 Polypeptide 1:

14394 14410 (MUC16 sequence)
NFSPLARRVDRVAIYEE (SEQ ID NO:01) 17 aa

Mouse monoclonals which are specific to this peptide are:
9B11.20.16 (IgG1)
10A2 (IgG1, IgM)
2F4 (IgG1, IgM)
23D3 (IgG1, IgG2b)
30B1 (IgG1)
31B2 (IgM)

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2. Muc16 Polypeptide 2:

14425 14442 (MUC16 sequence)
TLDRSSVLVDGYSPNRNE (SEQ ID NO:02) 18 aa

Mouse monoclonals which are specific to this peptide are:

4H11.2.5 (IgG2b)	13H1 (IgG1)	29G9 (IgG1)
9C9.21.5.13 (IgG2b)	28F8 (IgG1, IgM)	23G12 (IgG1, IgG2a)
9C7.6 (IgG1)	11B6 (IgM)	25G4 (IgG1, IgM)
5C2.17 (IgG1)	4C7 (IgG1)	26B2 (IgG1, IgG2b, IgM)
4A5.37 (IgG1)	4A2 (IgG1)	25H3 (IgG1, IgM)
28F7.18.10 (IgG1)		

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3. Muc16 Polypeptide 3 (SEQ ID NO:03)

14472 14492 (MUC16 sequence)
CGVLVTTRRRKKEGEYNVQQQ 21 aa

Mouse monoclonals which are specific to this peptide are:

31A3.5.1 (IgG1)	19D1 (IgG2b)	10F6 (IgG1)
22E10 (IgG2b)	22F1 (IgG2b, IgM)	3H8 (IgG1, IgM)
22F11 (IgM)	4D7 (IgG3)	24G12 (IgG1, IgM)
19G4 (IgG1, IgM)	9A5 (IgM)	
4C2 (IgG1, IgM)	31C8 (IgG2b)	
27G4 (IgM)	6H2 (IgG1, IgM)	

14452 14475
FWAVILIGLAGLLGLITCLICGVL (SEQ ID NO:14) is Transmembrane region 24 aa

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4. Muc16 Polypeptide 4 (SEQ ID NO:15) containing a cysteine loop polypeptide (SEQ ID NO:19):

14367 14398 (MUC16 sequence)
KSYFSDCQVSTFRSVPNRHHTGVDSLCNFSPL (SEQ ID NO:15) 32 aa
s - s

Mouse monoclonals which are specific to this peptide are:
24B3 (IgM)
9C7 (IgM)

4F12	IgM kappa
6H6	IgM kappa
25C2	IgM kappa
6E8	IgM kappa
2A3	IgM, IgG1, IgG2b, kappa
2G4	IgM, IgG1, kappa
4C8	IgM, kappa
2A6	IgG1 kappa
24G12	IgG1 kappa
15D5	IgG1 kappa
6E2	IgM, IgG1, IgG3, IgG2a, kappa
7E6	IgM, kappa, lambda
7G11	IgM kappa
20C3	IgG1, IgG2b
9A3	IgM kappa
15B6	IgM kappa
19D3	IgM kappa
5H8	IgM, IgG1, IgG2b, kappa
24A12	IgM kappa

2D10	IgG3, IgM kappa
5B2	IgM, IgG3, IgG2b, IgG2a, IgG1, kappa
8B6	IgG2a, IgG3, kappa
5A11	IgM, kappa
7D11	light kappa only
9F10	IgM, kappa
15D10	IgM, kappa
18D2	IgM, kappa
13A11	IgM, kappa

1A9	IgM, kappa
3B2	IgM, kappa
24F6	IgM, kappa
24E4	IgM, kappa
5A1	IgG2a, IgM, kappa
7B9	IgM, kappa
22F4	IgM, kappa

The identified monoclonal antibodies are listed in Table 1A and Table 2. Each of the selected monoclonal antibodies was reactive against GST-ΔMUC16^{c114}. The commercial MUC16-directed antibodies (OC125, M11, or VK8) did not bind to GST-ΔMUC16^{c114} in ELISA or Western blotting. The clones were tested in FACS against OVCAR3 ovarian cancer cells and in Western blot analysis against GST-ΔMUC16^{c114} (Table 1B), and selected purified monoclonal antibodies were isolated.

The inventors used the OVCAR3 wild type and the SKOV3 cells transduced with phrGFP-ΔMUC16^{c114} to characterize the selected antibodies by FACS analysis. All of the selected monoclonal antibodies bound to both cell lines while commercial VK8, M11 and OC125 antibodies bound to the OVCAR3 cells but not to the SKOV3-phrGFP-ΔMUC16^{c114} cell line. The antibodies against Polypeptide 3 required permeabilization since it is an internal epitope (Figure 7).

Western blot analysis using the GST-ΔMUC16^{c114} purified protein showed strong binding with 4H11 and 9C9 antibodies (Figure 4A), while the other selected antibodies showed less binding. The SKOV3-phrGFP-ΔMUC16^{c114} transfectant was also positive by Western blot analysis using 4H11 and 9C9 antibodies (Figure 4B). As before, the commercial antibodies did not interact with the GST-ΔMUC16^{c114} purified protein or cell lysates of the SKOV3-phrGFP-ΔMUC16^{c114} cell line.

The binding of six monoclonal antibodies against OVCAR3 MUC16 were examined in affinity binding studies. Three antibodies—9C7, 5C2 and 28F7—showed only modest levels of binding compared to the nonspecific binding of these antibodies to the OVCAR3 cells, which carry large numbers of MUC16 binding sites. In contrast, 4H11, 9C9, and 4A5 monoclonal antibodies showed highly specific binding affinity, as shown in Figure 5A, with binding affinities of 6.8-8.6 nM against the cell surface epitopes of OVCAR3 cells. The inventors also examined the internalization of antibody bound to cell surface MUC16 protein. The inventors examined internalization in the transfected SKOV3-phrGFP-ΔMUC16^{c334} cell line which bears the carboxy

terminus of MUC16, including the 4H11 epitope and a single degenerate tandem repeat sequence to interact with the OC125 antibody. The commercial antibodies OC125, M11, and VK8 all bind to the cell surface of this transduced cell line. The ^{131}I-labeled 4H11 showed rapid internalization at a high level, whereas ^{131}I-labeled OC125 antibody was internalized at a much lower rate (Figure 5B).

EXAMPLE 3

Immunohistochemistry results:

Given their highly specific binding affinities, the antibodies 9C9, 4A5, and 4H11 were characterized for utility in immunohistochemistry using OVCAR3 cell lines. Of the three, the 4H11 antibody was selected to be optimized for use in human tissues based on its robust, sensitive and specific staining pattern as compared to the other two antibodies.

A. Ovary

Two high-stage, high-grade ovarian serous carcinoma tissue microarray slides composed of 419 cores, representing primary, metastatic and recurrent tumors from 40 patients were stained with both OC125 and 4H11 monoclonal antibodies (Figure 2). The OC125 tissue microarrays showed 279 (66%) cores with 3-5 staining, 99 (24%) with 1-2 staining, and 41 (10%) with no staining. The 4H11 tissue microarrays showed 236 (56%) with 3-5 staining, 91 (22%) with 1-2 staining, and 92 (22%) with no staining. The two antibodies were concordant in 233 (56%) cores, equivocal in 161 (38%), and discordant in 25 (6%). Of the 25 discordant cores, 12 (48% of discordant cases, 3% of all cases) showed greater 4H11 positivity than OC125. Nine were discordant by a difference of 4 points, and 3 were discordant by a difference of 5 points. There was a total of 186 discordant and equivocal cores together, 48 (26%) of which showed greater staining with 4H11 than OC125. The staining pattern of both 4H11 and OC125 was cytoplasmic and membranous, although the membranous pattern of OC125 was stronger and better defined than 4H11 in the majority of cases. Discordant cases demonstrated higher levels of 4H11 than other cases.

B. Breast Cancer

A variety of other tissues were also examined for 4H11 staining to test the antibody's specificity. Of the 50 cores of invasive ductal carcinomas of the breast (number of patients unavailable), only 2 (4%) showed a score of 4 or greater 4H11 staining and none had scores of 3-5 for OC125 staining. The staining pattern with OC125 was mostly apical/luminal with some granular cytoplasmic staining. Some tumors with intracytoplasmic lumina also picked up the OC125 stain. 4H11 showed a more diffuse cytoplasmic blush without membranous accentuation.

In contrast, the invasive lobular breast carcinoma tissue microarray (composed of 179 cores with viable tumor, number of patients unavailable) had frequent MUC16 staining with 4H11. In this tissue microarray, 168 cores (94%) showed no staining for OC125, 5 (3%) showed 1-2 staining, and only 6 (3%) showed a staining intensity of 3. 4H11 staining was different in its distribution pattern, with 49 (27%) showing no staining, 81 (45%) showing 1-2 staining, and 49 (27%) showing 3-4 staining. Neither OC125 nor 4H11 had cores with a staining intensity of 5. The staining pattern was of cytoplasmic, luminal/membranous, or intraluminal for both OC125 and 4H11. The intraluminal pattern was strong and intense for both stains and highlighted the intracytoplasmic lumen that is commonly present in lobular carcinomas. The concordance rates were 34% concordant, 43% equivocal, and 23% discordant. Of the equivocal and discordant cases, there was none in which the OC125 was greater than the 4H11. All 42 discordant cases and 76 of 77 equivocal cases had 4H11 greater than OC125. There was also focal luminal staining with 4H11 in benign breast ducts and lobular carcinoma in situ.

C. Lung, pancreatic and prostatic adenocarcinomas

Tumors from other organs were not reactive with either antibody. The lung adenocarcinoma TMA had 237 cores from 86 patients containing viable tumor. In the pancreatic TMA there were 92 cores from 21 patients containing pancreatic mucinous tumors, including intraductal papillary mucinous neoplasms (IPMN) and invasive ductal carcinomas. In the prostate cancer TMA there were 169 cores (number of patients not available). None of these cancer tissue microarrays had significant binding to either OC125 or 4H11. This information is summarized in Table 3.

Table 3. Staining intensity of OC125 as compared to 4H11 in tissue microarrays

	OC125 vs. 4H11 staining intensity score (%)					
Site	**0**		**1-2**		**3-5**	
	OC125	**4H11**	**OC125**	**4H11**	**OC125**	**4H11**
Ovary high grade serous	10	28	24	22	66	56
Breast invasive ductal	68	78	32	18	0	4
Breast invasive lobular	94	27	3	45	3	27
Lung adenocarcinoma	63	77	24	18	13	5
Pancreas mucinous neoplasms	98	88	2	10	0	2
Prostate adenocarcinoma	0	0	0	0	0	0

Score 0: 0% staining; 1: <5% strong or weak; 2: 5-50% strong or weak; 3: 51-75% strong or 51-100% weak; 4: 76-99% strong; 5: 100% strong

D. **Normal Tissues**

There was no staining with OC125 or 4H11 in normal adult colon, rectum, ectocervix, small intestine, ovary, liver, pancreatic ducts, spleen, kidney, and skin. OC125 and 4H11 both stained endocervical glands (OC125 luminal, 4H11 weak cytoplasmic), esophageal glands (luminal), bronchial epithelium (OC125 luminal, 4H11 intracytoplasmic granules), and thymic corpuscles (cytoplasmic). 4H11 demonstrated weak to moderate staining of the gastric glands, particularly at the crypts, with an intracytoplasmic granular pattern. Other organs that showed punctuate intracytoplasmic staining with 4H11 only were prostate, seminiferous tubules of the testes, and the islet cells of the pancreas. The staining in the pancreatic islets cells was particularly strong and consistent. There was also nonspecific staining of liver, kidney and brain with 4H11. There were no cases that stained with OC125 and not 4H11.

Similarly, there was no staining with either OC125 or 4H11 in fetal heart, gallbladder, colon, small intestine, liver, rectum, adrenal, thyroid, spleen, skin, bone, epididymis, brain, lung, muscle, smooth muscle, kidney, eye, umbilical cord, and placenta. OC125 only stained thymic corpuscles in a pattern similar to that in adult tissue. 4H11 stained both fetal pancreatic endocrine cells and endocervical glands in a similar pattern to that of their adult counterparts. Islet cells showed a granular cytoplasmic pattern, and endocervical glands showed a linear luminal pattern, which was more similar to the OC125 pattern in the adult tissue.

EXAMPLE 4

Successful eradication of established peritoneal ovarian tumors in SCID-Beige mice following adoptive transfer of T cells genetically targeted to the MUC16 antigen.

Purpose: Most patients diagnosed with ovarian cancer will ultimately die from their disease. For this reason, novel approaches to the treatment of this malignancy are needed. Adoptive transfer of a patients own T cells, genetically modified *ex vivo* through the introduction of a gene encoding an chimeric antigen receptor (CAR), an artificial T cell receptor, targeted to a tumor associated antigen, is a novel and promising approach to cancer therapy applicable to the treatment of ovarian cancer.

Experimental design: We have generated several CARs targeted to the retained extracellular domain of MUC16, termed MUC-CD, an antigen highly expressed on a majority of ovarian carcinomas. We investigate the *in vitro* biology of human T cells retrovirally transduced to express these CARs by co-culture assays on artificial antigen presenting cells (AAPCs) generated from NIH3T3 fibroblasts genetically modified to express the target MUC-CD antigen, as well as by cytotoxicity assays utilizing the human OV-CAR3(MUC-CD) ovarian tumor cell line and primary

patient tumor cells. Finally, we assess the *in vivo* anti-tumor efficacy of MUC-CD targeted T cells in a SCID-Beige orthotopic, xenogeneic OV-CAR3(MUC-CD) murine tumor model.

Exemplary sequences used in this work are in Figure 17-19.

Results: CAR modified MUC-CD targeted T cells derived from both healthy donors and ovarian cancer patients exhibited efficient *in vitro* cytolytic activity against both human ovarian cell lines as well as primary ovarian carcinoma cells. MUC-CD targeted T cells may be further expanded *ex vivo* through multiple cycles of co-culture on 3T3(MUC-CD/B7.1) AAPCs. Expanded MUC-CD targeted T cells infused into SCID-Beige mice bearing intraperitoneal human OV-CAR3(MUC-CD) tumors either delayed progression or fully eradicated tumor even in the setting of advanced disease.

Conclusion: These promising pre-clinical studies justify further investigation of MUC-CD targeted T cells as a potential therapeutic approach in the clinical setting treating patients with high risk MUC-16^{+} ovarian carcinomas.

INTRODUCTION

Ovarian cancer is the sixth most common cancer worldwide and the seventh leading cause of cancer-related deaths in women (1, 2). Despite multimodality therapy with surgery and chemotherapy, most patients with ovarian carcinomas have a poor prognosis. For this reason, alternative approaches to treating this disease are urgently needed.

Infusion of a patient's own T cells genetically targeted *ex vivo* to antigens expressed on the surface of tumor cells is a promising novel approach to the adoptive immunotherapy of cancer, and one which has only recently been explored in earnest in the clinical setting. T cells may be genetically modified to target tumor associated antigens through the retroviral introduction of genes encoding artificial T cell receptors termed chimeric antigen receptors (CARs). Genetic engineering of T cells to express artificial T cell receptors that direct cytotoxicity toward a tumor cell presents a means to enhance immune recognition and elimination of cancer cells. CARs are most commonly composed of a single chain fragment length antibody (scFv), derived from a murine monoclonal antibody targeting a given tumor associated antigen, fused to a transmembrane domain (typically CD8, CD28, OX-40, and 4-1BB), fused to the TCR ζ chain cytoplasmic signaling domain (3-13). When used to reprogram T-cell specificity, these fusion receptors permit recognition of native antigen. When expressed by the T cells, the resulting construct, upon engagement with the targeted antigen, induces T cell activation, proliferation, and lysis of targeted cells. These fusion receptors transduce a functional antigen-dependent co-stimulatory signal in primary T cells, permitting sustained T-cell proliferation when both endogenous TCR and a chimeric receptor for stimulatory

signaling are engaged. To date, preclinical studies utilizing CAR-modified T cells have demonstrated promising results in a wide variety of malignancies (3, 4, 11, 14-18). More recently this approach been investigated clinically in the form of phase I trials (6, 19-21). These genetic approaches offer a means to enhance immune recognition and elimination of cancer cells.

Ovarian carcinomas appear to be relatively immunogenic tumors capable of inducing an endogenous immune response based on the fact that long-term prognosis of patients is markedly influenced by the degree and quality of the endogenous immune response to the tumor. Specifically, it has been well documented that the presence of endogenous effector T cells within the ovarian cancer tumor microenvironment directly correlates to prolonged patient survival (22-25). In contrast, increasing numbers of immune suppressive $CD4^+$ $CD25^{hi}$ regulatory T cells (Tregs) within the tumor, which in turn presumably abrogate the anti-tumor activity of infiltrating effector T cells, correlates with shorter patient survival (26-29). In fact, it appears that it is the ratio of Tregs to effector T cells within the tumor microenvironment which ultimately dictates whether the endogenous immune response to the cancer is of benefit or detriment to the patient (24, 28). In this setting, the ability to generate and subsequently expand a population of tumor targeted effector T cells *ex vivo* which are subsequently infused back into the patient, may in turn skew the Treg to effector T cell ratio to one more favorable to eradicating the disease.

Mucins are important biomolecules for cellular homeostasis and protection of epithelial surfaces. Changes to expression of mucins in ovarian cancer might be exploited in diagnosis, prognosis and treatment (1). MUC16 is one such mucin which is over expressed on most ovarian carcinomas and is an established surrogate serum marker (CA-125) for the detection and progression of ovarian cancers (30-33). MUC16 is a high-glycosylated mucin composed of a large cleaved and released domain, termed CA-125, consisting of multiple repeat sequences, and a retained domain (MUC-CD) which includes a residual non-repeating extracellular fragment, a transmembrane domain, and a cytoplasmic tail (34). Since the antigen is otherwise only expressed at low levels in the uterus, endometrium, fallopian tubes, ovaries, and serosa of the abdominal and thoracic cavities, MUC16 is a potentially attractive target for immune-based therapies.

However, the fact that most of the extracellular domain of MUC16 is cleaved and secreted limits the utility of MUC16 as a target antigen on ovarian carcinomas. In fact, to date, all reported MAbs to MUC16 bind to epitopes present on the large secreted CA-125 fraction of the glycoprotein, with none known to bind to the retained extra-cellular fraction (MUC-CD) of the antigen (35-37). Since the MUC-CD fraction of the antigen is retained on cell surface, generating T cells specific to this portion of MUC16 may largely overcome the limitation of MUC16 as a target for adoptive cellular immunotherapy. To this end, we have previously generated a series of murine

MAbs specific to the retained MUC-CD extracellular domain (38). Utilizing a hybridoma which expresses one such MAb, 4H11, we have successfully constructed several CARs specific to the MUC-CD antigen. This invention provides a nucleic acid encoding a chimeric T cell receptor, composed of, at least a zeta chain, a signaling region and a binding element that specifically interacts with a selected target as well as the chimeric T cell receptor comprising a zeta chain portion, a signaling region and a binding element.

In this report, we demonstrate highly efficient retroviral transduction of these MUC-CD targeted CARs into human T cells with resulting T cells able to specifically target and lyse MUC-CD$^+$ tumor cells *in vitro*. Furthermore, we demonstrate efficient MUC-CD targeted T cell expansion *in vitro* through repeated co-culture on NIH (3T3) fibroblasts genetically modified to express MUC-CD and the co-stimulatory ligand B7.1 (CD80). Successful expansion of modified T cells allowed us to subsequently generate sufficient T cell numbers to conduct *in vivo* studies in immune compromised SCID-Beige mice bearing established intraperitoneal MUC-CD$^+$ human ovarian tumors. Significantly, in these studies we demonstrate marked anti-tumor efficacy of MUC-CD targeted T cells, both following direct intraperitoneal as well as intravenous injection when compared to either untreated mice, or mice treated with T cells bearing a CAR targeted to an irrelevant antigen. In addition, we demonstrate significant cytotoxicity of 4H11-28z$^+$ patient's T cells and healthy donor's T cells targeting primary ascites-derived ovarian carcinoma cells from cancer patients.

To our knowledge this is the first report wherein T cells genetically targeted to the MUC16 antigen demonstrate marked anti-tumor efficacy against MUC16$^+$ tumors either *in vitro* or *in vivo*. These data serve as a rationale for proposing future clinical trials utilizing this approach in patients with high risk ovarian carcinomas.

MATERIALS AND METHODS

Cell lines and T cells

The OV-CAR3 tumor cell line was cultured in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FBS, nonessential amino acids, HEPES buffer, pyruvate, and BME (Invitrogen). The PG13 and gpg29 retroviral producer cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FCS, and NIH-3T3 artificial antigen-presenting cells (AAPC), described previously (3), were cultured in DMEM supplemented with 10% heat-inactivated donor calf serum. T cells were obtained from peripheral blood of healthy donors under IRB approved protocol #95-054, in BD Vacutainer® CPT tubes (Becton Dickinson, Franklin Lakes, NJ) as per the manufacturer's instructions. All media were supplemented with 2 mmol/L L-glutamine (Invitrogen),

100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). T cells were cultured RPMI 1640 media as above supplemented with 20 IU/ml IL-2 (Novartis Pharmaceuticals, East Hanover, NJ) and where indicated, medium was supplemented with 10 ng/mL interleukin 15 (R&D Systems, Minneapolis, MN).

Isolation of patients ascites-derived cancer cells

Primary human ascites-derived cancer cells were obtained from ovarian cancer patients undergoing surgery for newly diagnosed advanced serous ovarian carcinoma under IRB approved protocol #97-134. The tumor cells were isolated from ascitic fluid of patients by centrifugation at 600g for 10 min at room temperature. Cells were washed once with lx PBS and cultured in RPMI 1640 media supplemented with 10% FBS for future analysis.

Generation of the MUC-CD targeted 4H11z and 4H11-28z CARs

The heavy and light chain variable regions of the 4H11 monoclonal antibody were derived from the hybridoma cell line 4H11. Utilizing cDNA generated from 4H11 RNA we isolated the V_H coding region by RACE PCR utilizing modified primers as described elsewhere (39, 40). The V_L chain variable region was cloned by standard PCR utilizing modified primers as described by Orlandi et al (41, 42). The resulting V_H and V_L fragments were subcloned into the TopoTA PCR 2.1 cloning vector (Invitrogen) and sequenced. The V_H and V_L fragments were subsequently ligated to a $(Gly_4Ser)_3$ spacer domain, generating the 4H11 scFv and fused to the human CD8 leader peptide (CD8L) by overlapping PCR (9, 41). In order to construct the MUC-CD targeted 4H11 CARs, the coding region of the CD8L-4H11 scFv was fused to the human CD8 hinge and transmembrane domains (to generate the 4H11z CAR), or alternatively to the CD28 transmembrane and cytoplasmic signaling domains (to generate the 4H11-28z CAR), fused to the T cell receptor CD3-ζ signaling domain (3, 9, 43). The resulting CAR constructs were subsequently sub-cloned into the modified MMLV retroviral vector SFG (44). VSV-G preudotyped retroviral supernatants derived from transduced gpg29 fibroblasts were used to construct stable PG13 gibbon ape leukemia virus (GaLV) envelope-pseudotyped retroviral producing cell lines (41).

Retroviral gene transfer

Isolated healthy donor peripheral blood mononuclear cells (PBMCs) were activated with phytohemagglutinin (PHA) at 2µg/ml (Sigma. St. Louis, MO) and retrovirally transduced on retronectin coated non-tissue culture plates (45). Briefly, six-well non-tissue culture plates (BD Biosciences, San Jose, CA) were coated with RetroNectin® (RN) (Takara Biomedicals, Otsu, Japan)

as per manufacturer's instructions. Forty-eight hours after PHA activation, aliquots of 1×10^6 T cells in 1 ml of supplemented RPMI medium were placed in each well of the RN-coated plates, along with 1 ml of SFG retroviral supernatant. T cells were centrifuged daily for 3 consecutive days with fresh retroviral supernatant added daily at 2000g at 30°C for 1hr (45). Gene transfer was assessed on day 7 by FACS.

In order to generate the relevant NIH-3T3 murine fibroblast artificial antigen presenting cells, a MUC-CD construct encoding the retained extracellular, transmembrane and cytoplasmic domains of the MUC-16 antigen was initially subcloned into SFG retroviral vector, SFG(MUC-CD). 3T3(MUC-CD) AAPCs were generated by retroviral transduction of SFG(MUC-CD) into wild-type NIH-3T3 fibroblasts, while 3T3(MUC-CD/B7.1) AAPCs were generated by retroviral transduction of previously established 3T3(B7.1) fibroblasts (41, 46). Highly enriched cell lines were isolated by FACS.

To generate the OV-CAR3(MUC-CD) and OV-CAR3(MUC-CD/GFP-FFLuc) cell lines, we retrovirally transduced the WT OV-CAR3 human ovarian cancer cell line with SFG(GFP-FFLuc) as described previously (47) and/or SFG(MUC-CD) VSV-G pseudotyped retroviral supernatants derived from gpg29 fibroblasts as described elsewhere (44). Resulting tumor cells were sorted by FACS for either MUC-CD expression alone for the OVCAR3(MUC-CD) cell line, or dual MUC-CD and GFP expression for the OVCAR3(MUC-CD/GFP-FFLuc) cell line. MUC-CD expression by FACS was assessed using the 4H11 MAb.

In vitro analyses of CAR$^+$ human T cells

To assess *in vitro* expansion and cytokine release upon stimulation, transduced T cells were co-cultured for 7 days after retroviral transduction in 6-well tissue culture plates (BD Biosciences) on confluent NIH 3T3 AAPCs in RPMI medium supplemented with 10% FBS in the absence of supplemented cytokines. In order to generate sufficient numbers of CAR-modified T cells for *in vivo* studies, transduced T cells were co-cultured on B7.1$^+$ AAPCs (3T3(MUC-CD/B7.1)) in RPMI medium supplemented with 20 IU IL-2/mL and 10 ng/mL IL-15 as described previously (3, 43). Patients T cells were activated and expanded with human CD3/CD28 beads (DYNAL®, Invitrogen, Carlsbad, CA) following manufacturer's recommendations.

Western Blot analysis of CAR expression

Western blot analysis of T-cell lysates under reducing conditions with 0.1 mol/L DTT (Sigma) was performed as previously described (46). Briefly, transduced T cells were washed in PBS and resuspended in radioimmunoprecipitation assay (RIPA) buffer (Boston BioProducts,

Worcester, MA) with mini complete protease inhibitor as per the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Resulting proteins were separated on 12% SDS-PAGE mini gels (Bio-Rad, Hercules, CA) after the addition of 6X reducing loading buffer (Boston BioProducts, Worcester, MA) and heating at 100°C for 10 min. Separated proteins were subsequently transferred to Immobilon membranes and probed using an anti-human CD3ζ chain monoclonal antibody (BD Biosciences). Antibody binding was detected by probing the blot with goat anti-mouse horse radish peroxidase-conjugated antibody followed by luminescent detection using Western Lighting® Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) as per the manufacturer's instructions.

Cytotoxicity assays

In vitro modified T cell cytotoxicity was assessed using the DELFIA® EuTDA assay (PerkinElmer LAS, Inc, Boston, MA) following manufacturer's recommendations. Cytotoxocity was assessed at 2 hours at effector T cell to target OV-CAR3(MUC-CD) or primary tumor cells (E:T) at indicated ratios. Effector T cells in these assays represent the number of $CD8^+$ CAR* T cells.

Cytokine detection assays

Cytokine assays were performed as per manufacturer's specifications using a multiplex Human Cytokine Detection assay to detect IL-2 and IFNγ (Millipore Corporation, Billerica, MA) utilizing the Luminex IS100 system. Cytokine concentrations were assessed using IS 2.3 software (Luminex Corp., Austin, TX).

In vivo SCID-Beige mouse tumor models

In all *in vivo* studies, 8-12 week-old FOX CHASE C.B.-17 (SCID-Beige mice) (Taconic, Hudson, NY) were initially injected ip with either 3×10^6 OV-CAR3(MUC-CD), or for bioluminescent imaging (BLI) studies 3×10^6 OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells. Subsequently, 3×10^7 CAR^+ T cells were injected ip or iv on day 1 or 7 following tumor injection as indicated. Mice were monitored for distress as assessed by increasing abdominal girth, ruffled fur, and decreased response to stimuli. Distressed mice were euthanized. All murine studies were done in context of an Institutional Animal Care and Use Committee-approved protocol (#00-05-065).

Bioluminescent imaging (BLI) of OVCAR3(MUC-CD/GFP-FFLuc) tumor cells in SCID-Beige mice

BLI was performed using Xenogen IVIS imaging system with Living Image software (Xenogen; Alameda, CA). Briefly, OVCAR3(MUC-CD/GFP-FFLuc) tumor bearing mice were injected

by ip with D-luciferin (150 mg/kg; Xenogen) suspended in 200µl PBS and imaged under 2% isoflurane anesthesia after 10 min. Image acquisition was done on a 25-cm field of view at medium binning level for 0.5-min exposure time (3, 43).

Flow cytometry

All flow cytometric analyses of T cells and tumor cells was performed using a FACScan™ cytometer with Cellquest software (BD Biosciences). T cells were analyzed using CAR-specific polyclonal goat Alexa Fluor 647 antibody (Molecular probes, Eugene, OR) phycoerythrin-labeled anti-human CD4, CD8, B7.1 (Caltag Laboratories, Burlingame, CA), B7.2 (Invitrogen, Camarillo, CA), 4-1BBL, and OX40 antibodies (Ancell Corporation, Bayport, MN). 3T3(MUC-CD) and OV-CAR3(MUC-CD) cells were stained with Alexa Fluor® 647 labeled 4H11 antibody (generated and labeled in the MSKCC monoclonal antibody core facility).

CFSE labeling of CARP T cells

CAR$^+$ T cells were stained with CFSE using the CellTrace™ CFSE cell proliferation kit following manufacturer's recommendations (Molecular Probes, Eugene, OR). Proliferation of CFSE labeled T cells was analyzed by FACS. For detection of CFSE labeling T cells *in vivo*, ovarian tumors were macerated through 40 µm cell strainer (BD Falcon, Franklin Lakes, NJ) and washed twice with 2% FBS/PBS before antibody staining and FACS analysis.

Statistics

Survival data assessed by log-rank analysis using GraphPad Prism software (GraphPad Prism software, San Diego, CA). Cytokine data were analyzed by Student's one-tailed t-test.

RESULTS

We have constructed SFG retroviral vectors encoding first (4H11z) and second generation (4H11-28z) CARs targeted to the MUC-CD antigen using the 4H11 hybridoma which generates a MAb specific to the MUC-CD antigen (Figure 11A). We confirmed expression of appropriately sized CAR proteins by Western blot analysis of resulting PG-13 retroviral producer cells (SFG-4H11z and SFG-4H11-28z) probed with a ζ-chain specific antibody (data not shown).

In order to assess the function of the first generation 4H11z CAR, healthy donor T cells isolated from peripheral blood were retrovirally transduced to express the 4H11z and control 19z1

CARs (Figure 11B). Function of the 4H11z CAR was assessed by proliferation of 4H11z transduced T cells following co-culture on 3T3(MUC-CD/B7.1) AAPCs. Results demonstrate specific proliferation of 4H11z transduced T cells, when compared to 19z1 modified T cells (Figure 11C). These data are consistent 4H11z CAR mediated specific binding to the MUC-CD antigen and subsequent T cell activation.

Since most malignancies fail to express co-stimulatory ligands, we further modified the 4H11z CAR to express the CD28 transmembrane and cytoplasmic co-stimulatory signaling domains, constructing the second generation 4H11-28z CAR (Figure 11A). To assess whether the 4H11-28z CAR, when expressed by human T cells, was capable of generating both a primary activating signal (termed "signal 1") through the ζ chain, as well as a co-stimulatory signal (termed "signal 2") through the CD28 cytoplasmic domain, which in turn allows for efficient T cell proliferation in the absence of exogenous co-stimulatory ligands, we compared T cell proliferation following co-culture on either 3T3(MUC-CD) or 3T3(MUC-CD/B7.1) AAPCs in the absence of exogenous cytokines. As expected, the second generation 4H11-28z$^+$ T cells markedly expanded when compared to 4H11z$^+$ T cells upon co-culture with 3T3(MUC-CD) AAPCs. In contrast, both 4H11z$^+$ and 4H11-28z$^+$ T cells expanded similarly on 3T3(MUC-CD/B7.1) AAPCs (Figure 12A). Co-stimulation mediated by the 4H11-28z CAR was further verified by analysis of day 2 tissue culture supernatants from co-culture experiments on 3T3(MUC-CD) AAPCs demonstrating enhanced IL-2 secretion, a cytokine typically secreted in the context of T cell co-stimulation, when compared to control 19z1$^+$ and 19-28z$^+$ T cells and first generation 4H11z$^+$ T cells (Figure 12B). Secretion of IFNγ was comparable between 4H11z$^+$ and 4H11-28z$^+$ activated T cells.

We next assessed the ability of MUC-CD targeted T cells to expand following weekly re-stimulations through co-culture on 3T3(MUC-CD/B7.1) AAPCs in the context of exogenous IL-2 and IL-15 (3). Both 4H11z$^+$ and 4H11-28z$^+$ T cells expanded greater than 2 logs over 3 weeks (Figure 12C). T cells transduced with the 4H11-28z were further analyzed by FACS for CAR expression 7 days after initial activation on AAPCs and following two subsequent co-stimulations on AAPCs demonstrating an expected enrichment of the CAR$^+$ T cell fraction (Figure 12D). Similar data was generated with expanded 4H11z$^+$ T cells (data not shown).

In vitro cytotoxicity and proliferation of MUC-CD targeted T cells following co-culture with OV-CAR3(MUC-CD) and freshly isolated ascites derived ovarian tumor cells.

In order to assess the ability of 4H11z$^+$ and 4H11-28z$^+$ T cells to target and lyse human ovarian carcinoma tumors, we utilized the human OV-CAR3 cell line which was genetically modified to express the MUC-CD antigen thereby better reflecting the majority of clinical ovarian

tumor samples which express the 4H11-targeted MUC-CD antigen (48). We initially verified specific lysis by MUC-CD targeted T cells demonstrating similar significant cytotoxic activity of 4H11z and 4H11-28z CAR modified T cells targeting OV-CAR3(MUC-CD) tumor cells when compared control T cells expressing the irrelevant first and second generation CD19-targeted 19z1 and 1928z CARs (Figure 13A). Healthy donor T cells modified to express the 4H11-28z CAR similarly exhibited lysis of freshly isolated ascites derived MUC-CD$^+$ ovarian carcinoma cells when compared to 19-28z transduced T cells (Figure 13B). Moreover, patient's peripheral blood T cells modified to express the 4H11-28z CAR similarly lysed autologous primary MUC-CD$^+$ tumor cells derived from the same ascites sample when compared to T cells modified to express the control 19-28z CAR (Figure 13C).

We further assessed the ability of 4H11z$^+$ and 4H11-28z$^+$ T cells from healthy donors to proliferate following co-culture on OV-CAR3(MUC-CD) as assessed by FACS of CFSE labeled T cells, as well as absolute T cells numbers over 7 days following co-culture with tumor (Figures 13D and E). Surprisingly, we found that both 4H11z$^+$ and 4H11-28z$^+$ T cells expanded equally well following co-culture with OV-CAR3(MUC-CD) tumor cells suggesting the ability of this tumor cell line to co-stimulate T cells through expression of a co-stimulatory ligand. To address this possibility, we conducted further FACS analyses of OV-CAR3(MUC-CD) tumor cells demonstrating expression of the co-stimulatory 4-1BBL ligand (Figure 13F), but not the B7.1, B7.2, or OX-40L co-stimulatory ligands (data not shown).

In vivo anti-tumor activity of MUC-CD targeted T cells in SCID-Beige mice.

To assess the *in vivo* anti-tumor activity of 4H11z$^+$ and 4H11-28z$^+$ T cells, we next generated an orthotopic xenotransplant ovarian cancer tumor model by ip injection of OV-CAR3(MUC-CD) tumor cells into SCID-Beige mice. If left untreated, these mice developed marked ascites and multiple nodular peritoneal tumors by 3 weeks following tumor cell injection (Figure 14A). All untreated tumor bearing mice had to be euthanized by 7 weeks following tumor cell injection due to evidence of distress.

To assess the *in vivo* anti-tumor efficacy of MUC-CD-targeted T cells, SCID-Beige mice were injected ip with OV-CAR3(MUC-CD/GFP-FFLuc) tumor cells on day 1 followed by ip injection of 4H11z$^+$ or 4H11-28z$^+$ T cells on day 2. For negative controls, tumor bearing mice were either untreated or treated with T cells modified to express the irrelevant CD19-targeted CAR. Collectively, we found that 27% of all mice treated with MUC-CD targeted T cells (3/11 mice) remained alive without clinical evidence of disease 120 days out from tumor injection with no statistically significant difference in survival when comparing the 4H11z$^+$ and 4H11-28z$^+$ T cell

treated cohorts (Figure 14B). In contrast, both MUC-CD-targeted T cell treated cohorts demonstrated statistically significant enhanced survival when compared to untreated and $19z1^+$ T cell treated control cohorts.

To assess whether systemically infused MUC-CD-targeted T cells successfully traffic to ip tumors, we next compared ip to iv infusion of $4H11\text{-}28z^+$ T cells in SCID-Beige mice bearing ip OV-CAR3(MUC-CD/GFP-FFLuc) tumors. Both ip and iv $4H11\text{-}28z^+$ T cell treated mice exhibited statistically enhanced survival when compared to untreated or $19\text{-}28z^+$ T cell treated control cohorts as assessed by overall survival (Figure 15A) as well as by BLI of tumor progression (Figure 15B). Furthermore, we found overall survival between the ip and iv treated groups to be statistically equivalent by log rank analysis. These data imply successful trafficking of iv infused $4H11\text{-}28z^+$ T cells to peritoneal tumors. We further confirmed trafficking of iv infused CFSE labeled $4H11\text{-}28z^+$ T cells to the peritoneum by FACS analysis of single cell suspensions of macerated OV-CAR3(MUC-CD) tumors (Figure 15C).

In vivo anti-tumor activity of MUC-CD targeted T cells in SCID-Beige mice bearing well established OV-CAR3(MUC-CD/GFP-FFLuc) tumors.

To further assess whether $4H11\text{-}28z^+$ T cells were able to eradicate more clinically relevant tumor burdens, we next treated SCID-Beige mice bearing well established ip OV-CAR3(MUC-CD/GFP-FFLuc) tumor injected 7 days prior to adoptive T cell therapy. Once more, we found that therapy with MUC-CD targeted T cells markedly eradicated BLI evident disease in all treated mice (Figure 16A) with 5 of 8 treated mice eventually developing relapsed progressive disease, and 3 mice remaining disease free as assessed by BLI imaging (not shown) out to 120 days post-tumor cell infusion (Figure 16B). These data demonstrate potent *in vivo* anti-tumor activity mediated by MUC-CD targeted T cells even in the setting of advanced disease.

DISCUSSION

Based on extensive analyses of patient tumor samples, ovarian carcinomas appear to be relatively immunogenic tumors. Specifically, researchers have found there to be a direct correlation between prognosis following surgery and chemotherapy and the quantity of tumor infiltrating effector T cells (TILs) in pretreatment tumor samples (25, 49, 50). Furthermore, others have described an inverse correlation between prognosis following therapy and pre-treatment levels of Tregs within the tumor, which in turn presumably inhibit the anti-tumor function of tumor specific effector TILs (26, 28, 51). Both of these findings imply a role for an endogenous effector T cell response to tumor in controlling disease progression both prior to and following initial

therapy and strongly support the contention that ovarian carcinomas may be susceptible to killing by adoptive infusion of autologous T cells targeted to ovarian tumor cell antigens.

While endogenous effector TILs are one source for presumably tumor specific T cells, an alternative approach to adoptive T cell therapy is to isolate autologous peripheral blood T cells which in turn may be genetically modified *ex vivo* to target tumor cell antigens. One such genetic approach is to retrovirally transduce patient T cells with CARs targeted to surface exposed antigens either unique to or over-expressed by the tumor. To this end, promising preclinical studies utilizing this approach in other malignancies have recently been translated into the clinical setting (6, 16, 19, 52). Similarly, we have previously generated CARs targeted to the CD19 antigen expressed on normal B cells as well as most B cell malignancies and are currently conducting clinical trials treating patients with relapsed B cell chronic lymphocytic leukemia and acute lymphoblastic leukemias with autologous T cell modified to express a CD19 specific CAR (53).

Application of this approach to ovarian carcinomas requires the identification to suitable target antigens expressed on the tumor cell surface. Significantly, other investigators have studied this approach in both the pre-clinical and clinical setting (4, 11, 54-57). Specifically, several groups have demonstrated significant anti-tumor responses to subcutaneous human ovarian carcinoma cell line tumors in immune compromised mice following intratumoral and/or intravenous infusion of T cells expressing CARs specific to the mesothelin and Lewis-Y antigens overexpressed on these tumor cell lines (56, 58, 59). Furthermore, Kershaw et al recently published the results of a phase I clinical trial treating patients with relapsed ovarian carcinomas with autologous T cells modified to express a CAR specific to the alpha-folate receptor (6). The authors of this study found that therapy with targeted T cells was well tolerated, but noted a lack of anti-tumor response in these studies related to poor persistence of modified T cells over time as well as a yet undefined T cell inhibitory factor in the serum of several treated patients.

In our studies, we have chosen to target the MUC-16 glycoprotein which is over-expressed on a majority of ovarian carcinomas (1, 30, 32, 33). The utility of MUC-16 as a target antigen for adoptive T cell therapy is compromised by the fact that most of the extracellular portion of this molecule is cleaved by the tumor cell, secreted, and may be detected in the serum as the CA-125 tumor marker. However, following cleavage of this secreted fraction of MUC-16, there remains a residual extracellular fraction of the glycoprotein, termed MUC-CD, which is retained on the tumor surface and is therefore an attractive target for immune-based therapies. To this end, we utilized a series of murine hybridomas generated to the MUC-CD antigen to construct CARs specific to MUC-CD. Of these CARs, we identified a CAR generated from the 4H11 murine hybridoma termed 4H11z, which, when expressed in human T cells, following co-culture on 3T3(MUC-

CD/B7.1) AAPCs, resulted in rapid destruction of AAPC monolayers as well as marked modified T cell expansion. Significantly, the antigen to the 4H11 antibody is highly expressed on a majority of pre-treatment ovarian carcinoma surgical tumor samples obtained from patients treated at our institution as assessed by immuno-histochemistry (48).

Optimal T cell activation requires both a primary T cell receptor mediated signal, "signal 1," along with a co-stimulatory "signal 2." Classically, this co-stimulatory signal may be provided by ligation of either B7.1 (CD80) or B7.2 (CD86) on the target cell with the T cell co-stimulatory receptor CD28. Alternatively, co-stimulation may be generated by ligation of 4-1BBL or OX-40L on the target cell with the respective 4-1BB or OX40 co-stimulatory receptors on the T cell (12, 60, 61). Since most tumor cells fail to express co-stimulatory ligands, we and others have previously demonstrated that second generation CARs further incorporating the cytoplasmic signaling domains the co-stimulatory receptors CD28, 4-1BB, and/or OX40 resulted in CARs capable of providing both signal 1 and signal 2 to the T cell upon binding to cognate antigen in the absence of exogenous co-stimulatory ligands (7-10, 12, 13, 15, 16, 62-65). To this end, we constructed a second generation CAR from the 4H11z CAR incorporating the transmembrane and cytoplasmic signaling domain of CD28 as described elsewhere (3, 9, 43). Consistent with previous studies, we found that T cells transduced to express the resulting 4H11-28z CAR, but not the first generation 4H11z CAR, efficiently expanded upon co-culture with 3T3(MUC-CD) fibroblasts in the absence of exogenous co-stimulation consistent with the ability of the 4H11-28z CAR to deliver both signal 1 and signal 2 to the T cell. This conclusion is further supported by the finding that 4H11-28z$^+$ T cells secreted significantly higher levels of IL-2, a cytokine indicative of T cell co-stimulation, upon co-culture on 3T3(MUC-CD) fibroblasts when compared to T cells transduced to express the first generation 4H11z CAR.

We next assessed the ability of 4H11z$^+$ and 4H11-28z$^+$ T cells to target and lyse human ovarian carcinoma tumor cells. To this end, we initially utilized the OV-CAR3 human ovarian cancer cell line. Since the OV-CAR3 tumor cell line binds the 4H11 antibody weakly, we further genetically modified the cell line to express MUC-CD (OV-CAR3(MUC-CD)) to better mimic the clinical setting wherein a majority of clinical ovarian carcinoma tumor specimens highly express the 4H11 MUC-CD antigen (48). We demonstrated that human T cells modified to express either 4H11z or 4H11-28z eradicated OV-CAR3(MUC-CD) tumor cells *in vitro*, and surprisingly observed that both 4H11z$^+$ and 4H11-28z$^+$ T cells expanded following co-culture with tumor *in vitro*. To define the etiology of this unanticipated 4H11z$^+$ T cell expansion, we further assessed whether OV-CAR3(MUC-CD) tumor cells expressed co-stimulatory ligands, and found that this tumor cell line expressed 4-1BBL, consistent with our experimental findings as well as with

previously published reports demonstrating 4-1BBL expression by a variety of carcinoma cell lines (66-68). In order to further validate the clinical relevance of these findings, we subsequently demonstrated specific *in vitro* lysis of primary ascites-derived tumor cells isolated from untreated ovarian carcinoma patients by both healthy donor allogeneic 4H11-28z$^+$ T cells as well as more significantly autologous 4H11-28z$^+$ patient peripheral blood T cells. These data strongly support the contention that treatment with autologous 4H11-based CAR$^+$ T cells have promise in future clinical applications.

In order to assess the *in vivo* relevance of our *in vitro* findings, we next generated a murine orthotopic OV-CAR3(MUC-CD) tumor model in SCID-Beige mice. We injected mice i.p. with OV-CAR3(MUC-CD) tumor cells and the following day infused 4H11z$^+$, 4H11-28z$^+$, and control 19z1$^+$ T cells i.p. This treatment approach resulted in a significant but similar delay to tumor progression and long-term survival in both the 4H11z$^+$ and 4H11-28z$^+$ T cell treated cohorts when compared to untreated mice or mice treated with control T cells targeted to the irrelevant CD19 antigen. We next compared ip to iv treatment with 4H11-28z$^+$ T cells of orthotopic OV-CAR3(MUC-CD/GFP-FFLuc) bearing mice, and found similar statistically significant survivals of mice over time with either direct ip infusion of T cells or systemic iv infusion of targeted T cells. Significantly, iv treated mice by day 1 following treatment, exhibited successful trafficking of targeted T cells to the peritoneum. These data suggests that adoptive therapy with targeted T cells may be equally efficacious following either a direct infusion into the peritoneum or through systemic iv infusion. These findings further support the future clinical potential of this approach in treating patients both with local relapse of disease as well as metastatic relapse to sites outside of the peritoneum.

Finally, we assessed the ability of 4H11-28z$^+$ T cells to eradicate more established disease by delaying modified T cell ip infusion by 7 days, when mice had greater established tumor burdens as assessed by bioluminescent imaging. This experimental setting better reflects the initial clinical setting wherein this adoptive T cell approach would be utilized. Significantly, despite the setting of markedly established disease, 4H11-28z$^+$ T cells retained the ability to lyse larger tumor burdens, delay relapse of tumor, and in a significant percentage of mice, fully eradicate disease.

In the studies presented here, we have consistently utilized mixed populations of CD4$^+$ and CD8$^+$ CAR$^+$ T cells to assess both *in vitro* and *in vivo* anti-tumor activity. To this end, ongoing studies will address the role of isolated CD4$^+$ and CD8$^+$ CAR$^+$ T cell subsets in the successful eradication of disease in this SCID-Beige OV-CAR3(MUC-CD) tumor model. The results of these studies may have implications to translating this therapeutic approach to the clinical setting. Furthermore, we acknowledge the limitations associated with the presented SCID-Beige tumor

model. Namely, this is a xenotransplant model in an immune compromised mouse. To this end, ongoing studies in or laboratory are focused on generating a more clinically relevant syngeneic immune competent tumor model to better define the biology and anti-tumor efficacy of MUC-CD targeted CAR-modified T cells in the context of an intact immune system.

In conclusion, herein we present the first published data demonstrating the feasibility of targeting MUC-16, an antigen over-expressed on a majority of ovarian carcinomas, through adoptive therapy with genetically modified T cells targeted to the retained MUC-CD portion of the MUC-16 antigen. Further, this report is the first to demonstrate efficient targeting of T cells in an orthotopic, clinically relevant, murine model of ovarian cancer, demonstrating efficacy both by ip and iv infusion of modified T cells. Finally, these data support the further translation of this approach to the clinical setting in the form of a phase I clinical trial in patients with persistent or relapsed ovarian carcinomas following initial therapy with surgery and chemotherapy. [jn]

EXAMPLE 5

Raising Mouse MUC16 monoclonal antibodies in mice and hamsters.

We selected 3 different regions of mouse MUC16 genome for which monoclonal antibodies were generated in mouse and hamster. The selected regions of the mouse MUC16 are Peptide 1 (SEQ ID NO:21, ecto region of cytoplasmic domain), Peptide 2 (SEQ ID NO:22, first cysteine loop) and Peptide 3 (SEQ ID NO:23, second cysteine loop) (Figure 20A) and its comparison with human MUC16 is shown in Figure 20B. A cysteine was added to the peptide sequence at the N terminus of Peptide 1 (SEQ ID NO:21) and Peptide 3 (SEQ ID NO:23) for better conjugation with KLH. Individual peptides were conjugated to KLH using Promega kit. These 3 conjugated peptides were pooled and immunized into 5 mice and 4 hamsters. 5 immunizations with a 3 week interval for each immunization were administered. Sera from these animals were tested by ELISA for their specific reactivity with individual peptides (SEQ ID NO:21, 22 and 23). Positive selected animals were allowed to rest for a month and then i.v. boosted with pooled peptides immunogen (SEQ ID NO:21, 22 and 23) and harvested the spleens after 4 days. Splenocytes were mixed with hybridoma partners and plated into microtiter plates at various clonal densities. Plates were cultured at 37°C 5% CO_2 for 10 days and then selected the clones. Supernatants from these selected clones were tested by ELISA for their specific reactivity with individual peptides (SEQ ID NO:21, 22 and 23). Positive clonal sups were tested by FACS, western blot and imaging using 2 mouse cell lines (ID8 and BR5-FVB1) and a human cell line (OVCAR-3).

Table 4 shows the summary of mouse and hamster monoclonal antibodies against mouse MUC16 peptide antigens Peptide 1 (SEQ ID NO:21), Peptide 2 (SEQ ID NO:22), and Peptide 3 (SEQ ID NO:23). A very strong antigenic response was seen with Peptide 1 (SEQ ID NO:21).

Table 4

Mouse MUC16		Mouse mAbs	Frozen Mouse mAb	
Peptide 1		46	16 (3-IgG1; 8-IgG2b; 1-IgM; 4-Unkown isotype)	Animals not iv boosted with peptide 2
Peptide 2		0	0	
Peptide 3		6	6 (4-IgG1; 2-IgM)	
Peptide 1,2,3		0	0	
Peptide 1,2		0	0	
Peptide 2,3		0	0	
No Peptide		0	0	

Mouse MUC16		Hamster mAbs	Frozen Hamster mAb
Peptide 1		69	21
Peptide 2		6	6
Peptide 3		7	7
Peptide 1,2,3		2	1
Peptide 1,2		1	1
Peptide 2,3		1	0
No Peptide		10	2

Details of mouse and hamster mAbs against Peptide 1 (SEQ ID NO:21), Peptide 2 (SEQ ID NO:22), and Peptide 3 (SEQ ID NO:23 are listed in Table 5 and Table 6 respectively.

Table 5:

isotype	PEPTIDE	Fusion Well	Cloned	Clones			
-	1	**01D01**					
-	1	**09F07**					
IgG 1	1	**16A09**	**no success**				
-	1	**21A07**					
-	1	**24G10**					
IgG 1	1	**10C04**	**yes**	**10C4-3H5**	10C4-1F2	10C4-2H8	10C4-1G7
IgG 1	1	**17F02**	**yes**	**17F2-3G5**	17F2-3F6	17F2-2F9	17F2-1E11
IgG 2b	1	**01A08**					
IgG 2b	1	**01F08**					
IgG 2b	1	**12B10**	**yes**	12B10-3F7	**12B10-3G10**	12B10-2F6	12B10-2F10
IgG 2b	1	**17H10**					
IgG 2b	1	**18D05**					
IgG 2b	1	**23B12**					
IgG 2b	1	**25E09**		25E9-3	**25E9-5**	25E9-13	25E9-16
IgM	1	**16F12**					
IgG 1	[redacted]	**04A06**	**no success**				
IgG 1	[redacted]	**05D01**	**no success**				
IgG 1	[redacted]	**21B08**	**yes**	**21B8-1H11**	21B8-3G6	21B8-3H9	21B8-1G8
IgG 1	[redacted]	**21E01**	**yes**	21E1-1E3	21E1-1G9	21E1-2G7	21E1-3G12
IgM	[redacted]	**08A02**					
IgM	[redacted]	**13E05**					

Table 6:

Hamster mAb	Peptide	Cloned			
01H03					
02F02	1				
04E 4					
04G07	1				
04H01	3	4H1-2E1	4H1-2E3	4H1-3E1	4H1-3H3
06A08	1				
06F02	1				
07F08	3				
07H05	2				
09A05					
09E 1	3				
09F08	1				
09H10					
10G06	1				
10H11	1				
11B10	1				
12E09	2				
15A08	1	15A8-2E2	15A8-2E10	15A8-2E11	15A8-3D2
15H08	3				
19B05	1				
21H04	3				
22B05	2	22B5-1F6	22B5-3G9	22B5-2G8	22B5-3F11
22D11	3				
23G12	1				
25E 8	1				
27H09	3				
28B12	1&2&3				
28C12	2				
30H02	1				
31A11	2				
31C01	2				
33H06	1&2				
34F10	1				
34H05	1				
36C01	1				
36C11					
36E 4	1				
37E 10	1				
10H11	1				

Hamster antibody 22B05 recognizes mouse (SEQ ID NO:22) and also the corresponding human sequence (SEQ ID NO:15).

Western blot analysis using mouse ID8 and BR5-FVB1 cell extracts were also performed for all the selected monoclonal antibodies as shown in Figure 21 and Figure 22 respectively.

Among the mouse MUC16 monoclonal antibodies, we selected 12B10-3G10 subclone mouse mAb for further screening. Similarly, hamster monoclonal antibodies, 15A8-2E10, 22B5-2G8 and 4H1-2E1 subclones were selected for further screening.

Immunohistochemical analysis was performed with paraffin and cryosections of ID8 (mouse), OVCAR-3 (human), BR5-FVB1 (mouse) cell lines and 13.5 days of Embryo. Paraffin or cryosections were probed with mouse 12B10 mAb, hamster 15A8, hamster 22B5 and hamster 4E1 mAbs to see the early development of mouse MUC16 (Figure 23)

12B10-3G10 sub clone were further analyzed for single chain Fv fragments. Figure 24 shows 12B10-3G10 V_H and V_L DNA and Amino Acids sequences. Bioreactive supernatants and purified 12B10-3G10 were generated for animal studies and other characterization studies. FACS analysis was performed with purified 12B10-3G10 on ID8, OVCAR3 and BR5-FVB1 cells showing over 90% positivity to both mouse and human MUC16 ecto-domain fragment (Figure 25).

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The scope of the invention as defined by the attached claims should not be limited by the specific embodiments set forth in the examples, but should be given the broadest interpretation consistent with the specification as a whole.

SEQUENCE LISTING IN ELECTRONIC FORMAT

This description contains a sequence listing in electronic form in ASCII text format. A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

1. A monoclonal antibody or an antigen-binding fragment thereof, that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is:

CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:3).

2. The antibody or antigen-binding fragment thereof of claim 1, wherein the antibody is a chimeric antibody.

3. The antibody or antigen-binding fragment thereof of claim 1, wherein the antibody is a humanized antibody.

4. The antibody or antigen-binding fragment thereof of claim 3, wherein substantially all of the framework region residues of the humanized antibody are those of a human immunoglobulin sequence, and wherein one or more residues of the framework region are replaced by corresponding nonhuman residues.

5. The antibody or antigen-binding fragment thereof of any one of claims 1 to 4, wherein the antigen-binding fragment thereof is a Fab fragment, a F(ab')2 fragment, or a Fv fragment.

6. The antibody or antigen-binding fragment thereof of any one of claims 1 to 5, wherein the antibody lacks specific binding to a glycosylated MUC16 extracellular domain.

7. The antibody or antigen-binding fragment thereof of any one of claims 1 to 6, wherein the antibody, or antigen-binding fragment thereof, is covalently linked to a cytotoxic agent or a prodrug of a cytotoxic agent.

8. The antibody or antigen-binding fragment thereof of any one of claims 1 to 7, wherein the antibody internalizes into a cell.

9. The antibody or antigen-binding fragment thereof of any one of claims 1 to 8, wherein the antibody is an IgG.

Date Reçue/Date Received 2022-01-28

10. A single chain variable fragment (scFv) comprising a variable heavy (VH) chain and a variable light (VL) chain, wherein the VH chain and the VL chain are of a monoclonal antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03).

11. The scFv of claim 10 which is covalently linked to a cytotoxic agent or a prodrug of a cytotoxic agent.

12. A composition comprising (a) an antibody, or antigen-binding fragment thereof, of any one of claims 1 to 9, and (b) a pharmaceutically acceptable carrier.

13. A hybridoma cell that produces an antibody that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof, wherein the amino acid sequence of the MUC16 polypeptide is CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03).

14. Use of the antibody or antigen-binding fragment thereof of any one of claims 1 to 9, for identifying a subject as having a cancer in which MUC16 is expressed.

15. The use of claim 14, wherein the antibody or antigen binding fragment thereof is for administration to the subject to determine the presence and location of the antibody in the subject, wherein the antibody is labeled.

16. The use of claim 14 or 15, wherein the cancer is ovarian cancer or breast cancer.

17. The antibody or antigen binding fragment thereof of any one of claims 1 to 9, for use in identifying a subject as having a cancer in which MUC16 is expressed.

18. The antibody or antigen binding fragment thereof for use of claim 17, wherein the antibody is for administration to the subject to determine the presence and location of the antibody in the subject, wherein the antibody is labeled.

19. The antibody or antigen binding fragment thereof for use of claim 17 or 18, wherein the cancer is ovarian cancer or breast cancer.

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20. An *ex vivo* method for identifying a subject as having a cancer in which MUC16 is expressed, wherein said method comprises:

(a) contacting a sample obtained from the subject with the antibody or antigen binding fragment thereof, of any one of claims 1 to 9; and

(b) detecting an increased level of binding of the antibody or antigen binding fragment thereof to the sample as compared to a control sample lacking the cancer in which MUC16 is expressed.

21. The *ex vivo* method of claim 20, wherein the cancer is ovarian cancer or breast cancer.

22. The *ex vivo* method of claim 20 or 21, wherein the detecting is selected from the group consisting of immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), Western blot, immunoprecipitation, and radiographic imaging.

23. Use of a polypeptide of the sequence:

CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03);

for producing an antibody or antigen binding fragment thereof that specifically binds to a MUC16 polypeptide or to an antigenic portion thereof.

24. Use of an antibody or antigen binding fragment thereof as defined in any one of claims 1 to 9, for treating a cancer.

25. Use of an antibody or antigen binding fragment thereof as defined in any one of claims 1 to 9, for formulating a medicament for treating cancer.

26. Use of a scFv, as defined in claim 10 or 11, for treating a cancer.

27. Use of a scFv, as defined in claim 10 or 11, for formulating a medicament for treating a cancer.

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28. The use according to any one of claims 24 to 27, wherein the cancer is ovarian cancer or breast cancer.

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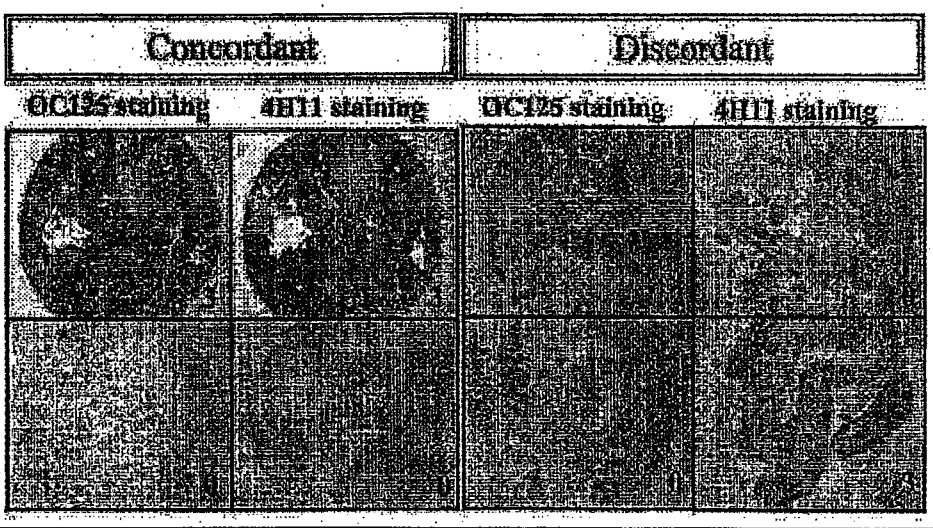
Peptide 1 near Cleavage Site:
NFSPLARRVDRVAIYEE (SEQ ID NO:01)

Peptide 2 before Transmembrane:
TLDRSSVLVDGYSPNRNE (SEQ ID NO:02)

Peptide 3 inside Transmembrane:
CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03)

FIGURE 1



Tissue microarrays were scored from 0 to 3 in one-half (+) increments for cytoplasmic and/or membranous staining
0 – no staining
1 – strong or weak <5%
1+ - strong or weak 5-50%
2 – strong 51-75% or weak 51-100%
2+ - strong (76-99%)
3 – strong (100%)

	Stain score		
n=518	0	1-1+	2-3
OC125	60 (11%)	122 (24%)	336 (65%)
4H11	129 (25%)	116 (22%)	273 (53%)

Concordance between OC125 and 4H11 staining was based on the amount of difference between the scores
Concordant = 0 - ½ difference
Partially concordant = 1-1 ½ difference
Discordant = 2-3 difference

OC125/4H11 (n=518)	
Concordant	286 (55%)
Partially concordant	193 (37%)
Discordant	39 (8%)

Of the 39 discordant cases,
OC125 > 4H11 in 59%
4H11 > OC125 in 41%

OC125>4H11	23 (59%)
4H11>OC125	16 (41%)

Overall 3-4% of all ovarian serous carcinomas expressed 4H11 but not OC125

FIGURE 2

FIGURE 3A

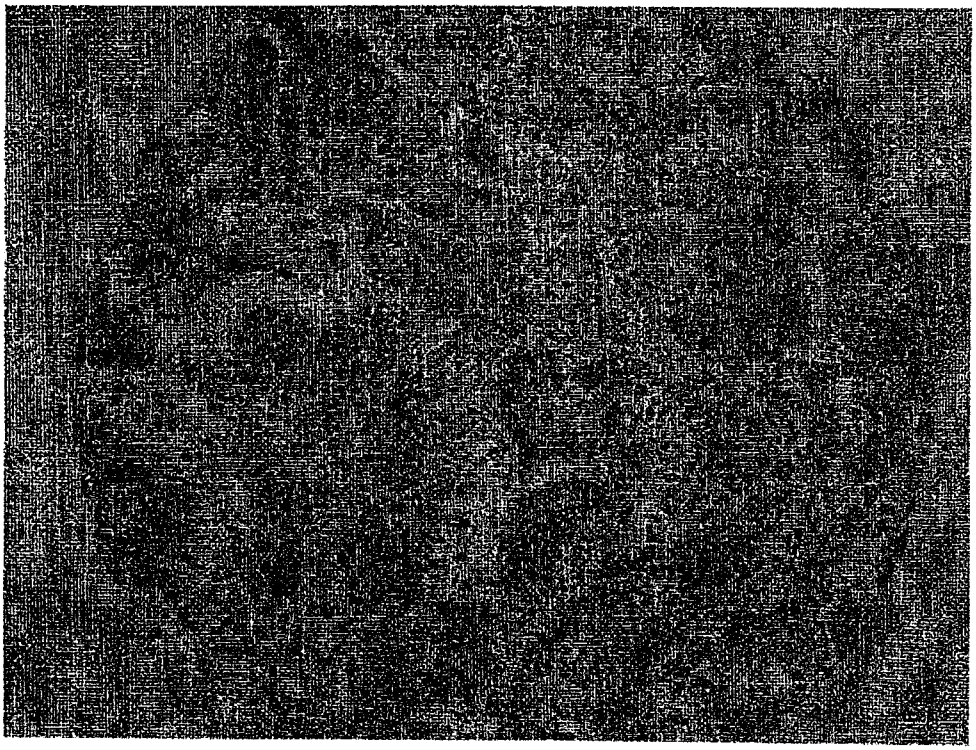


FIGURE 3B

FIGURE 3C

FIGURE 3D

FIGURE 3E

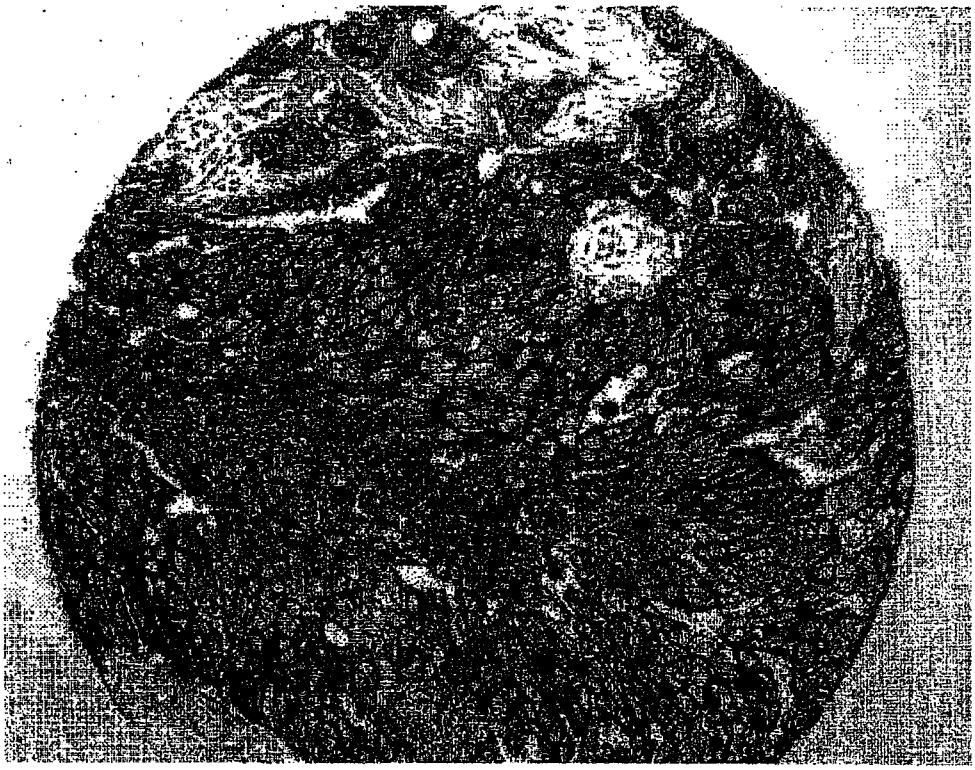


FIGURE 3F

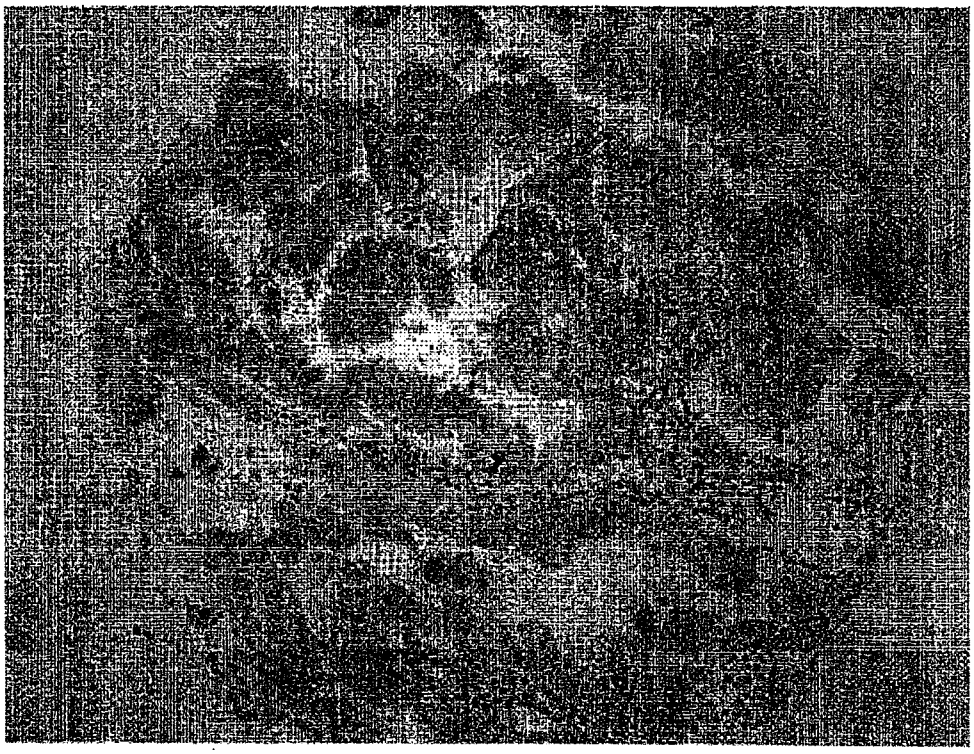


FIGURE 3G

FIGURE 3H

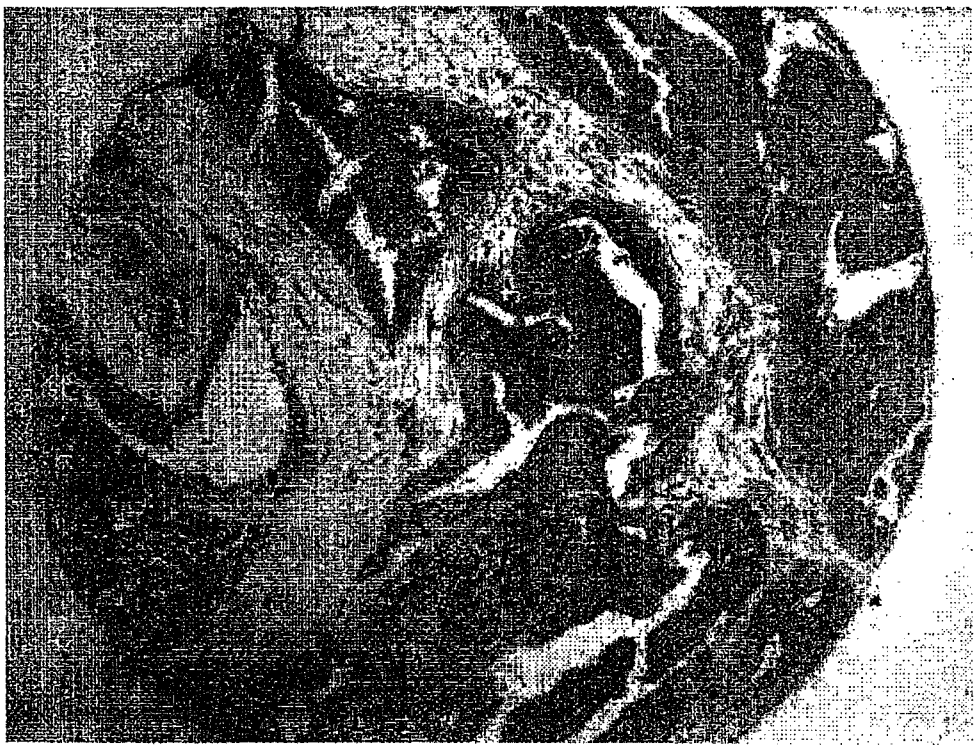


FIGURE 3I

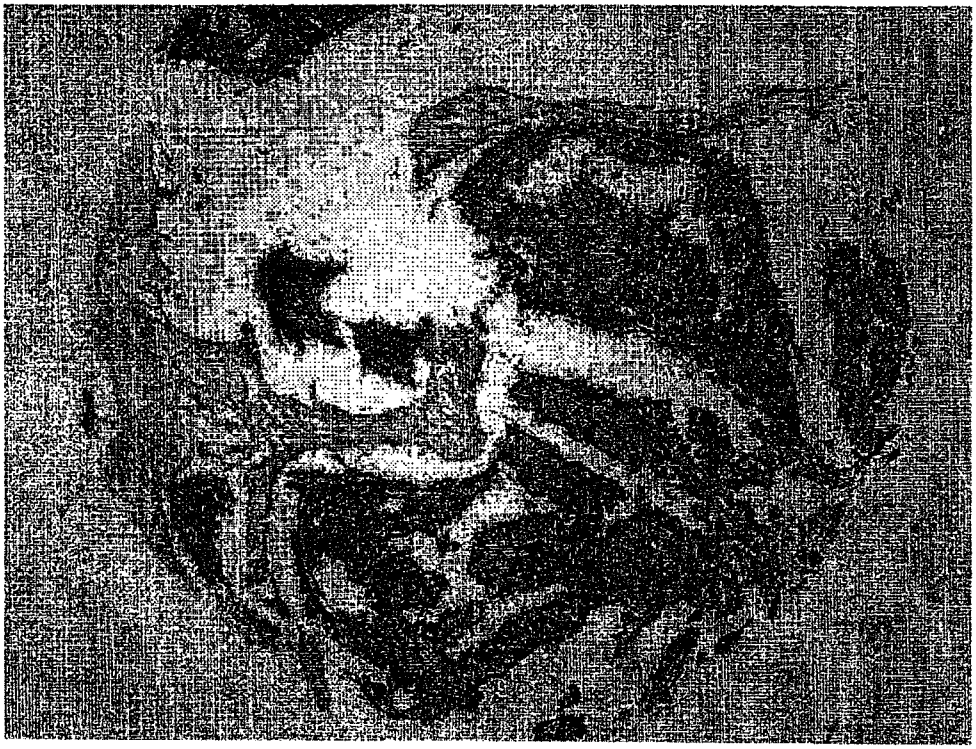


FIGURE 3J

FIGURE 3K

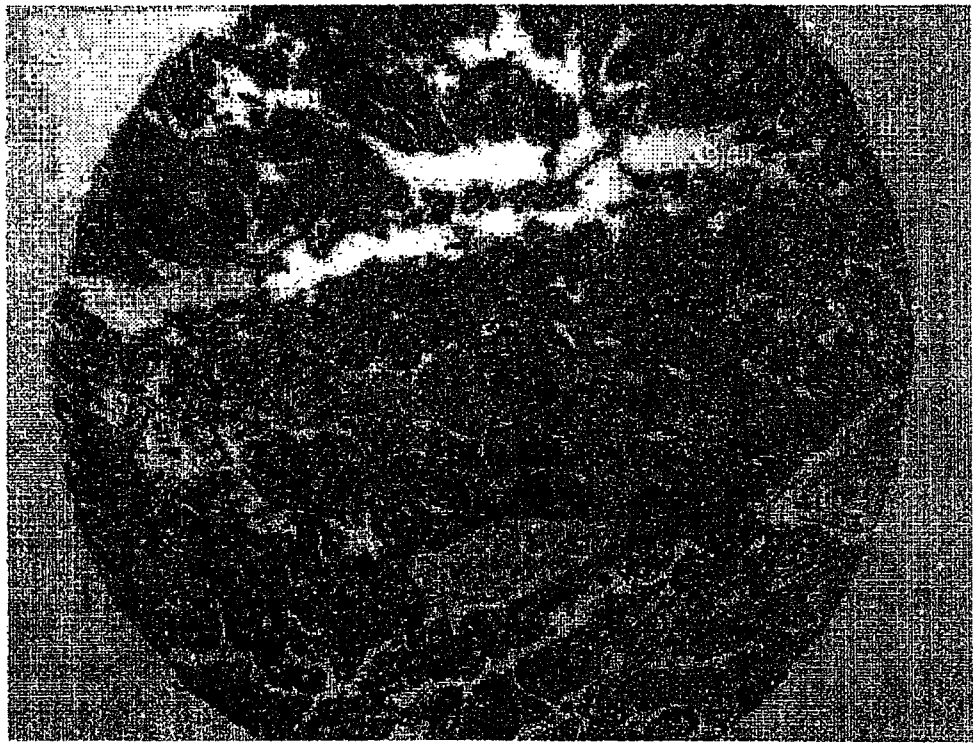


FIGURE 3L

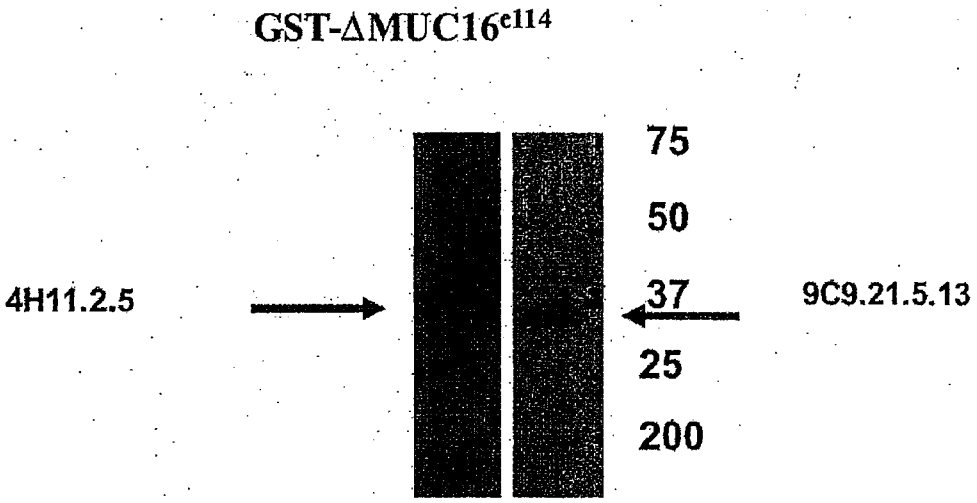


FIGURE 4A

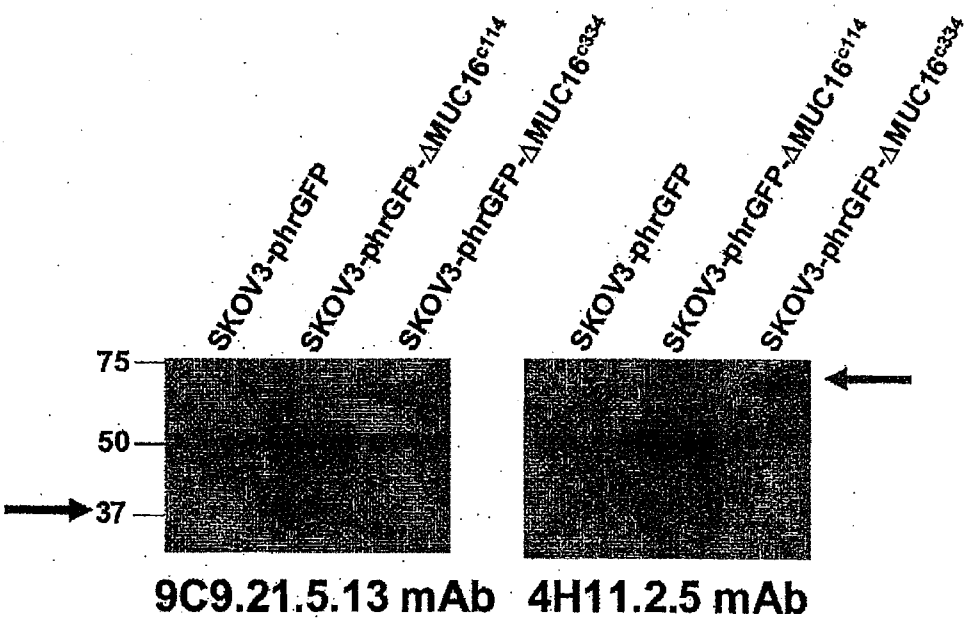

SKOV3-phrGFP
SKOV3-phrGFP-ΔMUC16c114
SKOV3-phrGFP-ΔMUC16c334
SKOV3-phrGFP
SKOV3-phrGFP-ΔMUC16c114
SKOV3-phrGFP-ΔMUC16c334
75
50
37
9C9.21.5.13 mAb
4H11.2.5 mAb

FIGURE 4B

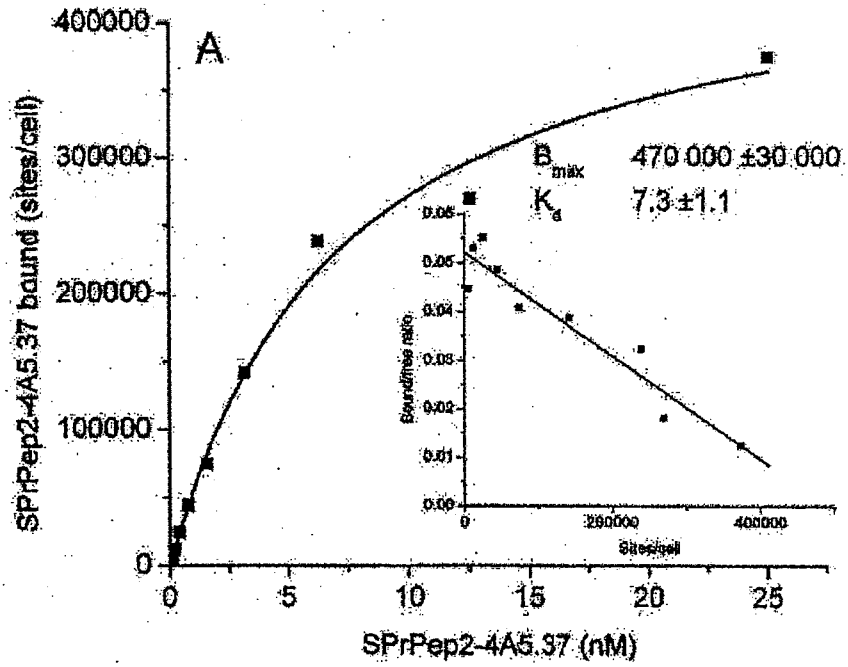


FIGURE 5A, PANEL A

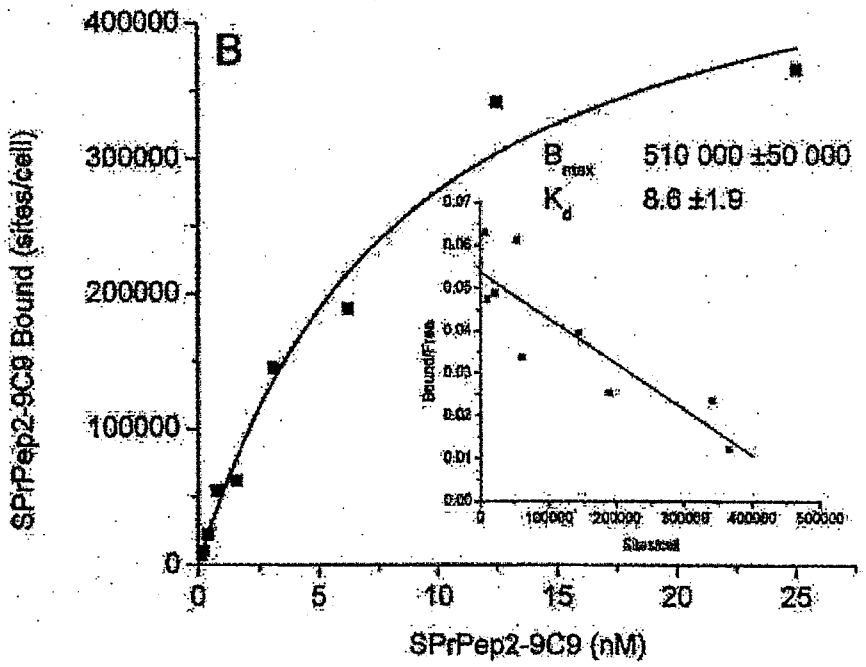


FIGURE 5A, PANEL B

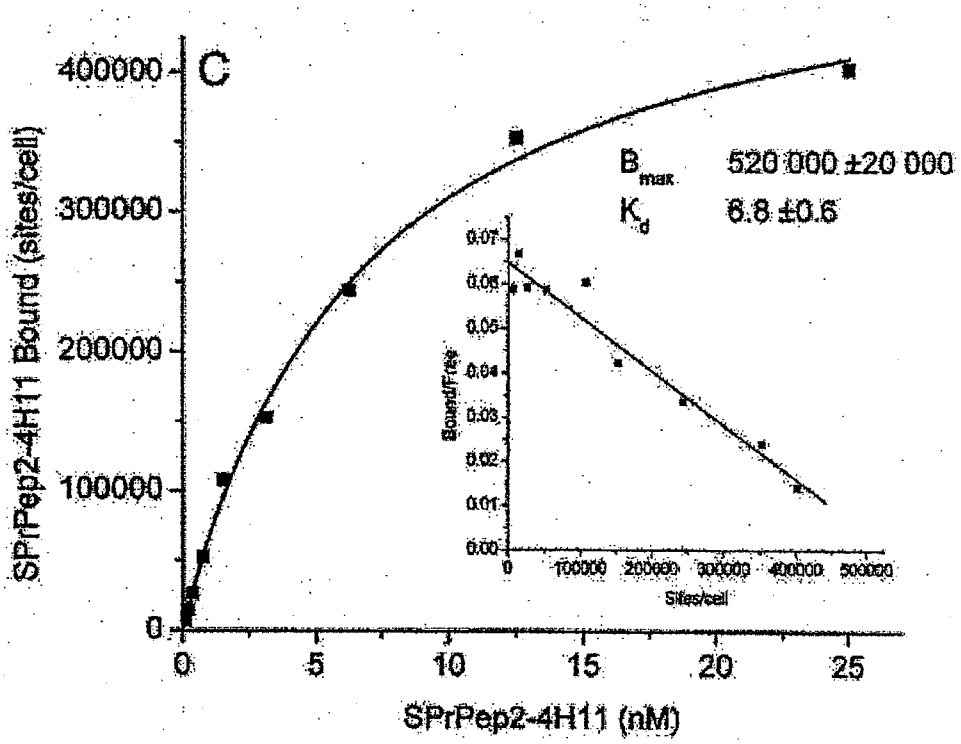


FIGURE 5A, PANEL C

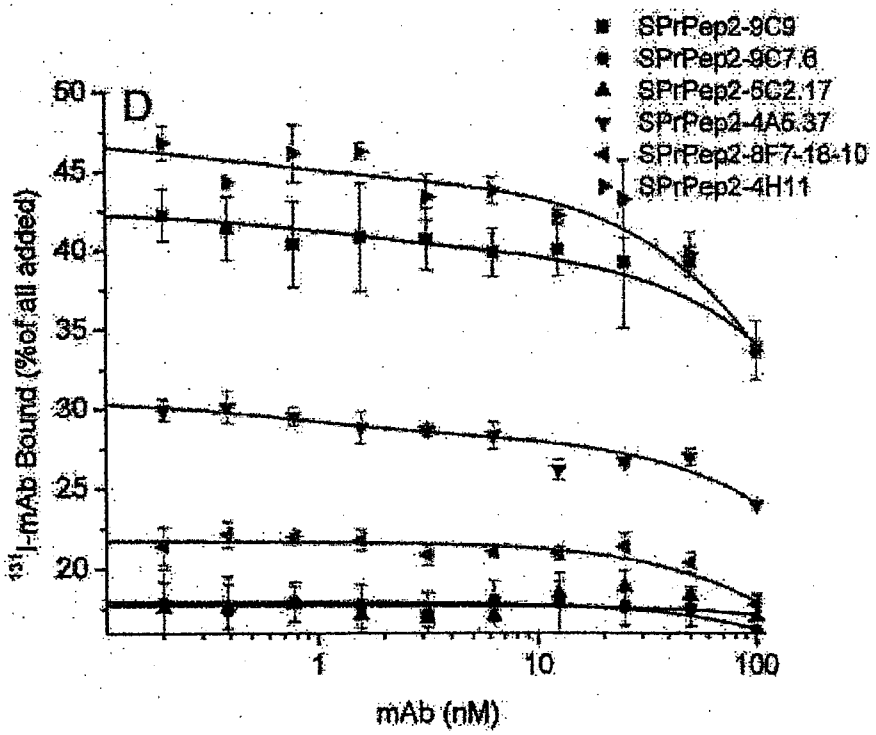


FIGURE 5A, PANEL D

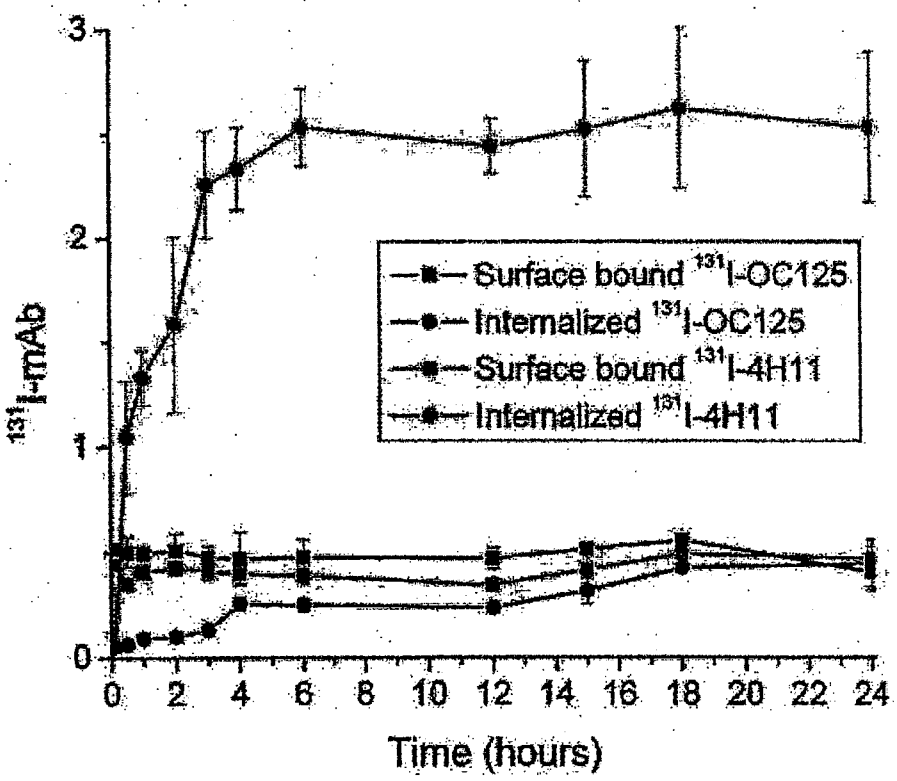

3
2
1
0
^{131}I-mAb
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)
Surface bound ^{131}I-OC125
Internalized ^{131}I-OC125
Surface bound ^{131}I-4H11
Internalized ^{131}I-4H11

FIGURE 5B

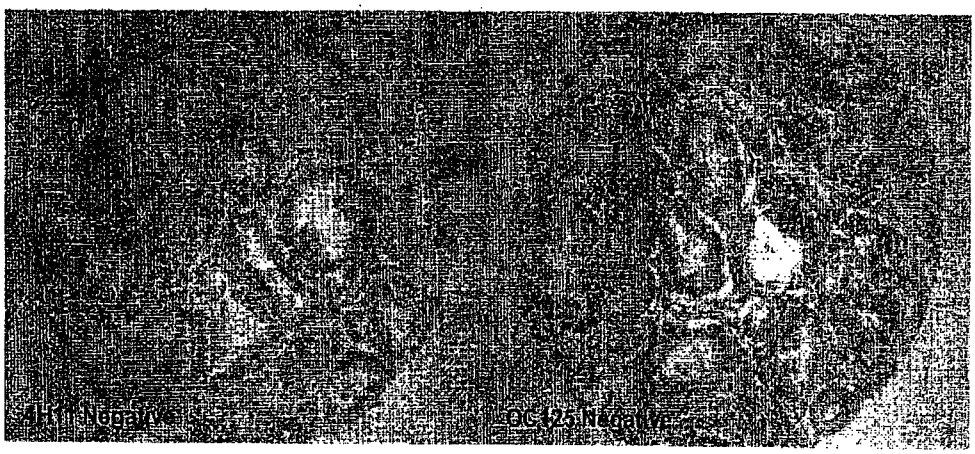


FIGURE 6A

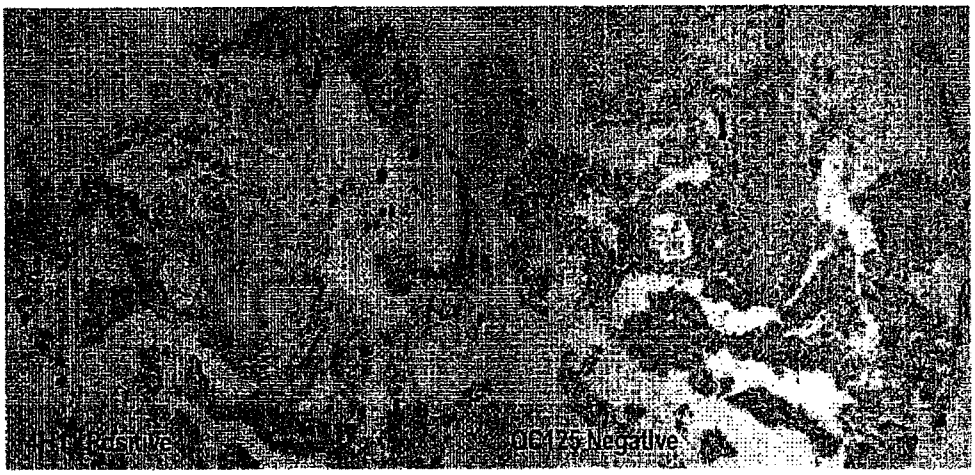


FIGURE 6B

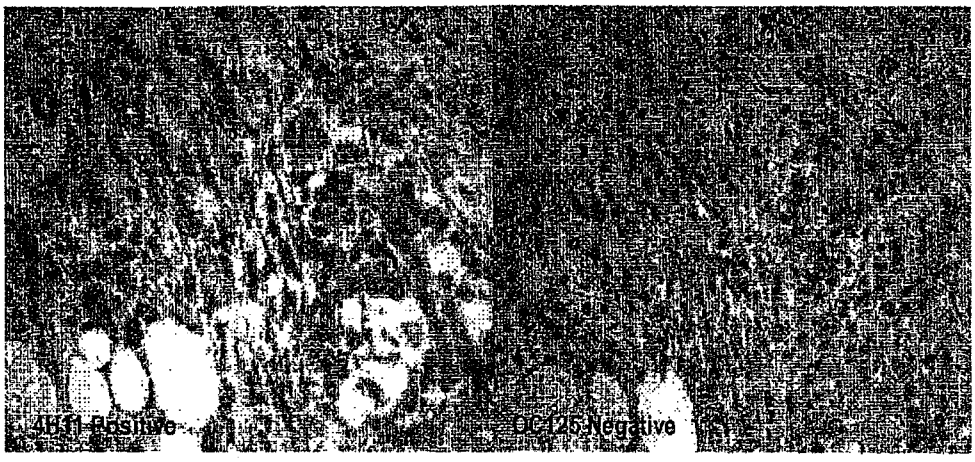


FIGURE 6C

FIGURE 6D

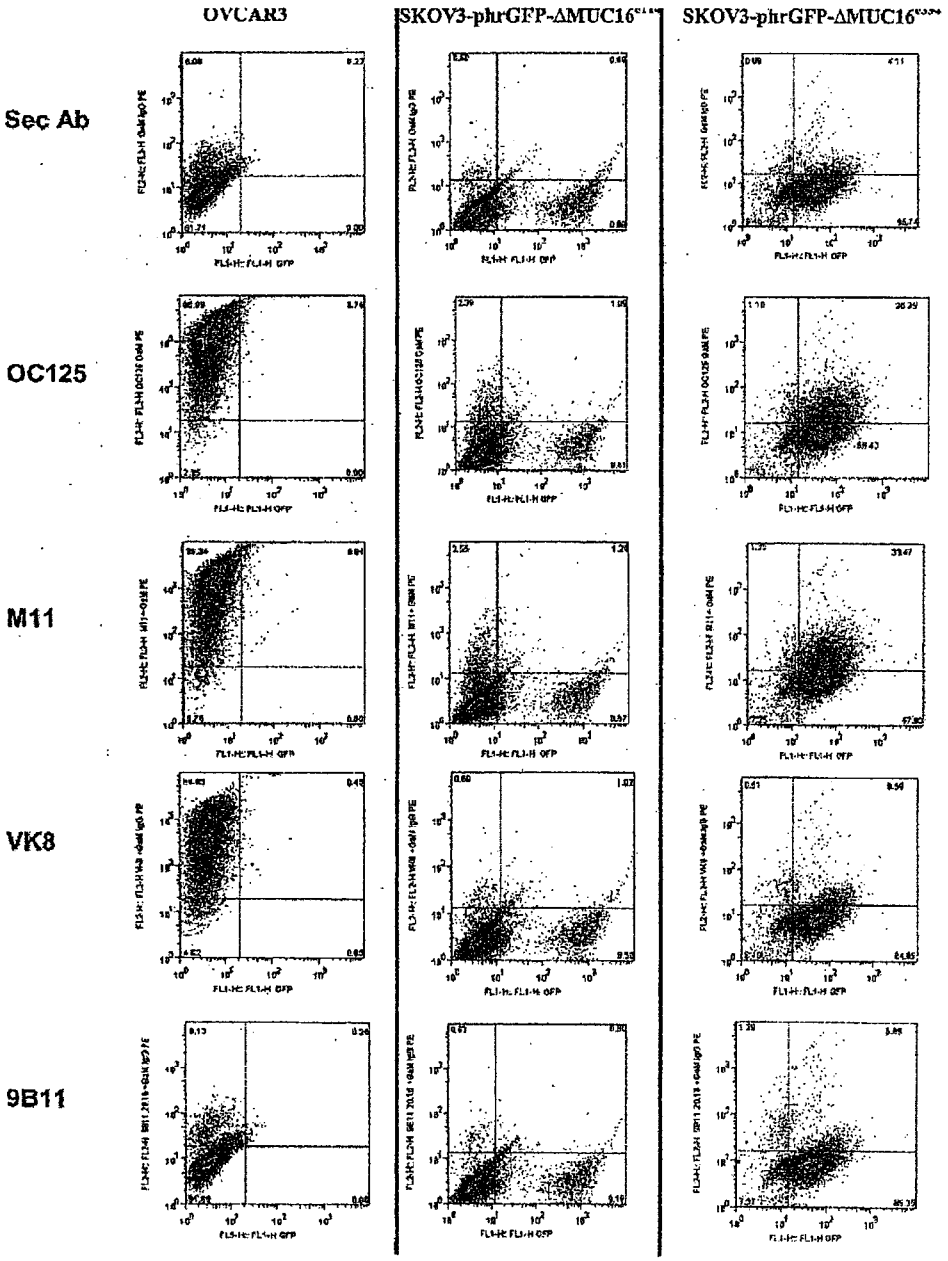


FIGURE 7, PAGE 1 OF 2

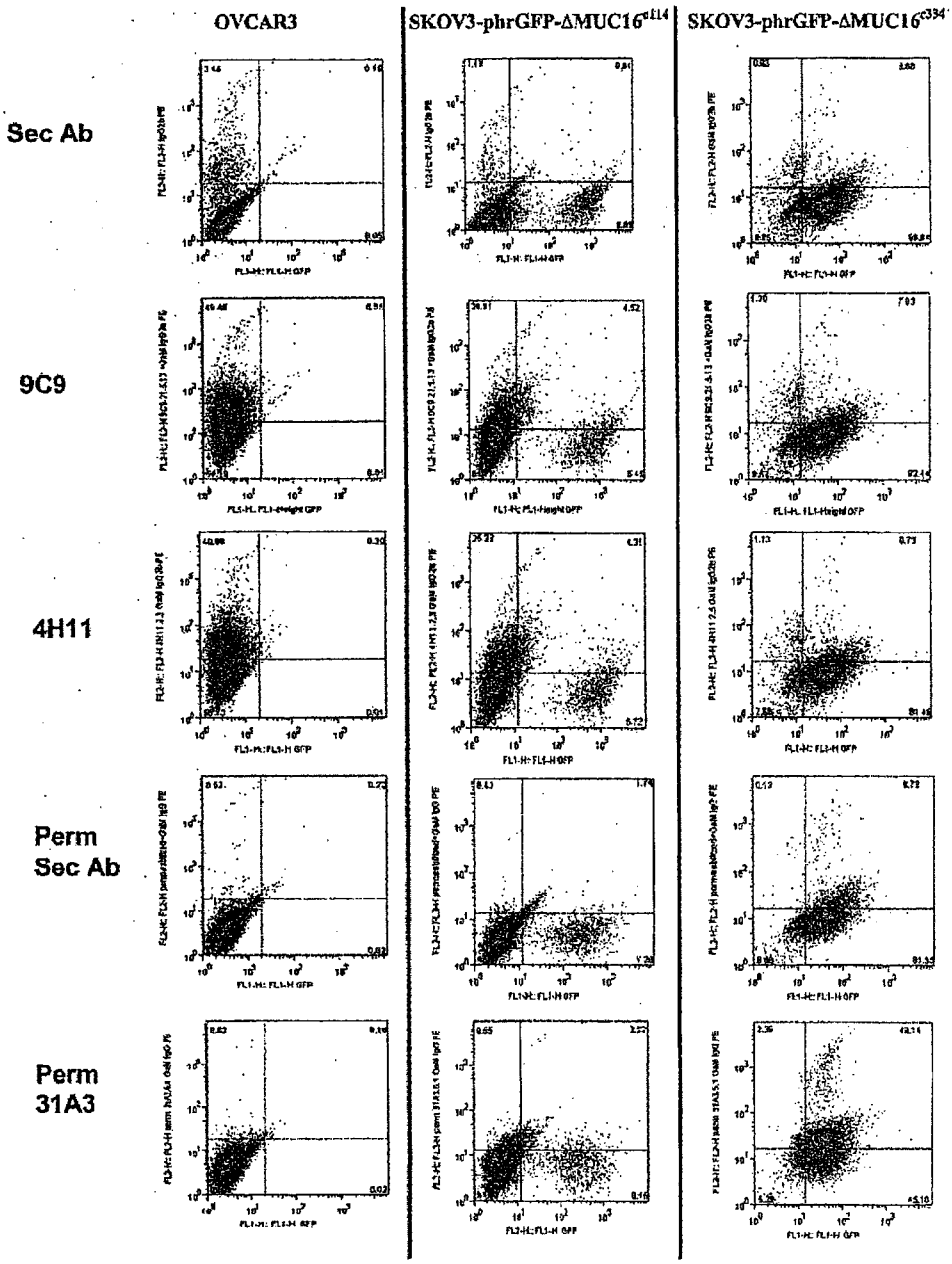


FIGURE 7, PAGE 2 OF 2

(A) 4A5 VH (SEQ ID NO:04)
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tttcagaaactatgccatgtcctgggttcgcctgagtccggagatgaggctggagtgggtcgcaaccattagcagtgctg
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caaatgggcagtctgaggtctggggacacggccatgtattactgtgcaaggcagggatttggtaactacggtgattacta
tgctatggactactggggccaagggaccacggtcaccgtctcctca

(B) 4A5 VL (SEQ ID NO:05)
gacattgagctcacccagtctccatcctccctggctgtgtcagcaggagagaaggtcactatgagctgcaaatccagtca
gagtctgctcaacagtagaacccgaaagaaccagttggcttggtaccagcaaaaaacaggacagtctcctgaactgctga
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atcagcagtgtgcaggctgaagacctggcagtttattactgccagcaatcttataatctactcacgttcggtcctgggac
caagctggagatcaaacgg

(C) 4H11 VH (SEQ ID NO:06)
gtgaagctgcaggagtcagggggaggcttcgtgaagcctggagggtccctcaaagtctcctgtgcagcctctggattcac
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gtggttacatcttctattctgacagtgtgcagggacgattcaccatttccagagacaatgccaagaacaccctgcacctg
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tgctatggactactggggccaagggaccacggtcaccgtctcctca

(D) 4H11 VL (SEQ ID NO:07)
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gagtctgctcaacagtagaacccgaaagaaccagttggcttggtaccagcaaaaaccaggacagtctcctgaactgctga
tctactgggcatccactaggcaatctggagtccctgatcgcttcacaggcagtggatctgggacagatttcactctcacc
atcagcagtgtgcaggctgaagacctggcagtttattactgccagcaatcttataatctactcacgttcggtcctgggac
caagctggaggtcaaacgg

(E) 9B11 VH (SEQ ID NO:08)
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ccaagggaccacggtcaccgtctcctca

(F) 9B11 VL.A (SEQ ID NO:09)
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caagctggaggtcaaacgg

(G) 9B11 VL.B (SEQ ID NO:10)
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aaggttcacatgttccgtggacgttcggtggagggaccaagctggagatcaaacgg

FIGURE 8 (1 of 2)

(H) 24B3-VH (SEQ ID NO:11)
GAGGTGAAGCTGGAGGAGTCAGGACCTGAACTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGTTA
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ACAATGGTGCTACTTTCTACAATCAGAAGTTCACGGGCAAGGCCACAATGACTGTAGACAAATCCTCTACCACAGCCCAC
ATGGAGCTCCTGAGCCTGACATCTGAGGACTCTGCAGTCTATTATTGTGGAAAGGGGAATTACTACGGCCCCTTTGATTA
CTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA

(I) 24B3-VL (SEQ ID NO:12)
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GAGCATTAGCAAATATTTAGCCTGGTATCAAAAGAAACCTGGGAAAACTAATAAGCTTCTTATCTACTCTGGATCCACTT
TGCAATCTGGAATTCCATCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTTCACTCTCACCATCAGTAGCCTGGAGCCT
GAAGATTTTGCAATGTATTACTGTCAACAGCATAATGAATACCCGTGGACGTTCGGTGGAGGGACCAAGCTGGAGATCAA
ACGGGCGGCCGCA

FIGURE 8 (2 of 2)

(A) Homo sapiens MUCIN-16 (GenBank NP_078966) (SEQ ID NO:13)

```
   1 mlkpsglpgs ssptrslmtg srstkatpem dsgltgatls pktstgaivv tehtlpfts
  61 dktlasptss vvgrttqslg vmssalpest srgmthseqr tspslspqvn gtpsrnypa
 121 smvsglsspr trtsstegnf tkeastytlt vettsgpvte kytvptetst tegdstetp
 181 dtryipvkit spmktfadst askenapvsm tpaettvtds htpgrtnpsf gtlyssfld
 241 spkgtpnsrg etslelilst tgypfsspep gsaghsrist saplsssasv ldnkisets
 301 fsgqsltspl spgvpearas tmpnsaipfs mtlsnaetsa ervrstissl gtpsistkq
 361 aetiltfhaf aetmdipsth iaktlasewl gspgtlggts tsaltttsps ttlvseetn
 421 hhstsgkete gtlntsmtpl etsapgeese mtatlvptlg fttldskirs psqvssshp
 481 relrttgsts grqssstaah gssdilratt sstskasswt sestaqqfse pqhtqwvet
 541 psmkterppa stsvaapitt svpsvvsgft tlktsstkgi wleetsadtl igestagpt
 601 hqfavptgis mtggsstrgs qgtthlltra tassetsadl tlatngvpvs vspavskta
 661 gssppggtkp sytmvssvip etsslqssaf regtslgltp lntrhpfssp epdsaghtk
 721 stsipllssa svledkvsat stfshhkats sittgtpeis tktkpssavl ssmtlsnaa
 781 spervrnats plthpspsge etagsvltls tsaettdspn ihptgtltse ssespstls
 841 psvsgvkttf ssstpsthlf tsgeeteets npsvsqpets vsrvrttlas tsvptpvfp
 901 mdtwptrsaq fssshlvsel ratsstsvtn stgsalpkis hltgtatmsq tnrdtfnds
 961 apqsttwpet sprfktglps atttvstsat slsatvmvsk ftspatssme atsirepst
1021 ilttettngp gsmavastni pigkgyiteg rldtshlpig ttassetsmd ftmakesvs
1081 svspsqsmda agsstpgrts qfvdtfsddv yhltsreiti prdgtssalt pqmtathpp
1141 pdpgsarstw lgilssspss ptpkvtmsst fstqrvttsm imdtvetsrw nmpnlpstt
1201 ltpsniptsg aigkstlvpl dtpspatsle asegglptls typestntps ihlgahass
1261 spstikltma svvkpgsytp ltfpsiethi hvstarmays sgsspemtap getntgstw
1321 pttyitttdp kdtssaqvst phsvrtlrtt enhpktesat paaysgspki ssspnltsp
1381 tkawtitdtt ehstqlhytk laekssgfet qsapgpvsvv iptsptigss tleltsdvp
1441 eplvlapseq ttitlpmatw lstslteema stdldissps spmstfaifp pmstpshel
1501 kseadtsair ntdsttldqh lgirslgrtg dlttvpitpl tttwtsvieh stqaqdtls
1561 tmspthvtqs lkdqtsipas aspshltevy pelgtqgrss seattfwkps tdtlsreie
1621 gptniqstpp mdntttgsss sgvtlgiahl pigtsspaet stnmalerrs statvsmag
1681 mgllvtsapg rsisqslgrv ssvlsestte gvtdsskgss prlntqgnta lsslepsy
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1801 gstentgkek lrtasmdlps ptpsmevtpw isltlsnapn ttdsldlshg vhtssagtl
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1981 eqstqplhav ssekvsvqtq stpyvnsvav saspthensv ssgsstsspy ssaslesld
2041 tisrrnaits wlwdlttslp tttwpstsls ealssghsgv snpsstttef plfsaasts
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2161 sestanpslg tassagtklt rtislptses lvsfrmnkdp wtvsiplgsh pttntetsi
2221 vnsagppgls tvasdvidtp sdgaesiptv sfspspdtev ttishfpekt thsfrtiss
2281 theltsrvtp ipgdwmssam stkptgasps itlgerrtit saapttspiv ltasftets
2341 vsldnettvk tsdildarkt nelpsdssss sdlintsias stmdvtktas isptsisgm
2401 assspslfss drpqvptstt etntatspsv ssntysldgg snvggtpstl ppftithpv
2461 tssallawsr pvrtfstmvs tdtasgenpt ssnsvvtsvp apgtwtsvgs ttdlpamgf
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2581 ptsganwets atpesllvvt etsdttltsk ilvtdtilfs tvstppskfp stgtlsgas
2641 ptllpdtpai pltateptss latsfdstpl vtiasdslgt vpettltmse tsngdalvl
2701 tvsnpdrsip gitiqgvtes plhpsstsps kivaprntty egsitvalst lpagttgsl
2761 fsqssenset talvdssagl erasvmpltt gsqgmassgg irsgsthstg tktfsslpl
2821 mnpgevtams eittnrltat qstapkgipv kptsaesgll tpvsasssps kafasltta
2881 ptwgipqstl tfefsevpsl dtksaslptp gqslntipds dastasssls kspeknpra
2941 mmtstkaisa ssfqstgfte tpegsaspsm agheprvpts gtgdpryase smsypdpsk
3001 ssamtstsla sklttlfstg qaarsgssss pislsteket sflsptasts rktslflgp
3061 marqpnilvh lqtsaltlsp tstlnmsqee ppeltssqti aeeegttaet qtltftpse
3121 ptsllpvssp teptarrkss petwassisv paktslvett dgtlvttikm ssqaaqgns
3181 wpapaeetgs spagtspgsp emsttlkims skepsispei rstvrnspwk tpettvpme
3241 tvepvtlqst algsgstsis hlptgttspt ksptenmlat ervslspspp eawtnlysg
3301 pggtrqslat mssvslespt arsitgtgqq sspelvsktt gmefsmwhgs tggttgdth
3361 slstssnile dpvtspnsvs sltdkskhkt etwvsttaip stvlnnkima aeqqtsrsv
3421 eaysstssws dqtsgsditl gaspdvtntl yitstaqtts lvslpsgdqg itsltnpsg
3481 ktssassvts psigletlra nvsavksdia ptaghlsqts spaevsildv ttaptpgis
3541 tittmgtnsi stttpnpevg mstmdstpat errttstehp stwsstaasd swtvtdmts
3601 lkvarspgti stmhttsfla ssteldsmst phgritvigt slvtpssdas avktetsts
3661 rtlspsdtta stpistfsrv qrmsisvpdi lstswtpsst eaedvpvsmv stdhastkt
3721 pntplstflf dslstldwdt grslssatat tsapqgattp qeltletmis patsqlpfs
3781 ghitsavtpa amarssgvtf srpdptskka eqtstqlptt tsahpgqvpr saattldvi
3841 htaktpdatf qrqgqtaltt earatsdswn ekekstpsap witemmnsvs edtikevts
3901 ssvlrtlntl dinlesgtts spswksspye riapsesttd keaihpstnt vettgwvts
3961 ehashstipa hsasskltsp vvttstreqa ivsmstttwp estrartepn sfltielrd
4021 spymdtsstt qtsiisspgs taitkgprte itsskrisss flaqsmrssd spseaitrl
4081 nfpamtesgg milamqtspp gatslsaptl dtsataswtg tplattqrft ysekttlfs
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4321 atetstvith vssgdatthv tktqatfssg tsissphqfi tstntftdvs tnpstslim
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4561 pstatspmvp assmgdalas isipgsettd iegeptsslt agrkenstlq emnsttesn
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4741 easspssqap vlvttlpssv aftpqwhsts spvsmssvlt sslvktagkv dtsletvts
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5101 sdkilatskd skdtkeifps inteetnvka nnsgheshsp aladsetpka ttqmvittt
5161 gdpapstsmp vhgssettni kreptyfltp rlretstsqe ssfptdtsfl lskvptgti

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5401 erpatykdta hteaamhhst ntavtnvgts gsghksqssv ladsetskat plmsttstl
5461 dtsvststpn isqtnqiqte ptaslsprlr esstsektss ttetntafsy vptgaitqa
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6001 ntetihfsen taetnmgttn smhklhssvs ihsqpsghtp pkvtgsmmed aivststpg
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6541 ltslptsglv kttdmlasva slppnlgsts hkipttsedi kdtekmypst niavtnvgt
6601 tsekesyssv payseppkvt spmvtsfnir dtivstsmpg sseitrieme stfslahgl
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7201 adsetskats pmfitstqed ttvaistphf letsriqkes isslspklre tgssvetss
7261 ietsavlsev sigatteisr tevtsssrts isgsaestml peisttrkii kfptspila
7321 ssemtiktqt sppgstsest ftldtsttps lvithstmtq rlphseittl vsrgagdvp
7381 psslpveets ppssqlslsa mispspvsst lpasshsssa svtslltpgq vkttevlda
7441 aepetsspps lsstsveila tsevttdtek ihpfsntavt kvgtsssghe spssvlpds
7501 ttkatsamgt isimgdtsvs tltpalsntr kiqsepassl ttrlretsts eetslatea
7561 tvlskvstga ttevsrteai sfsrtsmsgp eqstmsqdis igtiprisas svltesakm
7621 ittqtgpses tlestlnlnt attpswveth siviqgfphp emttsmgrgp ggvswpspp
7681 vketsppssp lslpavtsph pvsttflahi ppsplpvtsl ltsgpatttd ilgtstepg
7741 sssssslstts herlttykdt ahteavhpst ntggtnvatt ssgyksqssv ladsspmct
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7981 tspspvsstl pesissplp vtslltsgla kttdmlhkss epvtnspanl sstsveila
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8101 stpgffetsr iqteptsslt lglrktssse gtslatemst vlsgvptgat aevsrtevt
8161 ssrtsisgfa qltvspetst etitrlptss imtesaemmi ktqtdppgst pesthtvdi
8221 ttpnwveths tvtqrfshse mttlvsrspg dmlwpsqssv eetssassll slpattsps
8281 vsstlvedfp saslpvtsll npglvittdr mgisrepgts stsnlsstsh erlttledt
8341 dtedmqpsth tavtnvrtsi sghesqssvl sdsetpkats pmgttytmge tsvsistsd
8401 fetsriqiep tssltsglre tssserissa tegstvlsev psgattevsr tevissrgt
8461 msgpdqftis pdisteaitr lstspimtes aesaitietg spgatsegtl tldtstttf
8521 sgthstaspg fshsemttlm srtpgdvpwp slpsveeass vssslsspam tstsffstl
8581 esisssphpv talltlgpvk ttdmlrtsse petssppnls stsaeilats evtkdreki
8641 pssntpvvnv gtviykhlsp ssvladlvtt kptspmatts tlgntsvsts tpafpetmm
8701 qptssltsgl reistsqets satersasls gmptgattkv srtealslgr tstpgpaqs
8761 ispeisteti tristplttt gsaemtitpk tghsgassqg tftldtssra swpgthsaa
8821 hrsphsgmtt pmsrgpedvs wpsrpsvekt sppsslvsls avtspsplys tpsesshss
8881 lrvtslftpv mmkttdmldt slepvttspp smnitsdesl atskatmete aiqlsenta
8941 tqmgtisarq efyssypglp epskvtspvv tsstikdivs ttipasseit riemestst
9001 tptpretsts qeihsatkps tvpykaltsa tiedsmtqvm sssrgpspdq stmsqdist
9061 vitrlstspi ktestemtit tqtgspgats rgtltldtst tfmsgthsta sqgfshsqm
9121 almsrtpgdv pwlshpsvee assasfslss pvmtsspvs stlpdsihss slpvtsllt
9181 glvkttellg tssepetssp pnlsstsaei laitevttdt eklemtnvvt sgythesps
9241 vladsvttka tssmgitypt gdtnvltstp afsdtsriqt kskslsltpgl metsiseet
9301 satekstvls svptgattev srteaisssr tsipgpaqst mssdtsmeti tristpltr
9361 estdmaitpk tgpsgatsqg tftldsssta swpgthsatt qrfpqsvvtt pmsrgpedv
9421 wpsplsvekn sppsslvsss svtspsplys tpsgsshssp vpvtslftsi mmkatdmld
9481 slepettsap nmnitsdesl aaskattete aihvfentaa shvettsate elyssspgf
9541 eptkvispvv tsssirdnmv sttmpgssgi trieiesmss ltpglretrt sqditsste
9601 stvlykmpsg atpevsrtev mpssrtsipg paqstmsldi sdevvtrlst spimtesae
9661 tittqtgysl atsqvtlplg tsmtflsgth stmsqglshs emtnlmsrgp eslswtspr
9721 vettrssssl tslplttsls pvsstlldss pssplpvtsl ilpglvktte vldtssepk
9781 ssspnlssts veipatseim tdtekihpss ntavakvrts ssvheshssv ladsettit
9841 psmgitsavd dttvftsnpa fsetrripte ptfsltpgfr etstseetts itetsavly
9901 vptsattevs mteimssnri hipdsdqstm spdiitevit rlssssmmse stqmtittql
9961 sspgataqst ltlatttapl arthstvppr flhsemttlm srspenpswk sslfvekts
10021 sssllslpvt tspsvsstlp qsipsssfsv tslltpgmvk ttdtstepgt slspnlsgt
10081 veilaasevt tdtekihpss smavtnvgtt ssghelyssv sihsepskat ypvgtpssm
10141 etsistsmpa nfettgfeae pfshltsgfr ktnmsldtss vtptntpssp gsthllqssl
10201 tdftssakts spdwppasqy teipvdiitp fnaspsites tgitsfpesr ftmsvtestl
10261 hlstdllpsa etistgtvmp slseamtsfa ttgvpraisg sgspfsrtes gpgdatlst
10321 aeslpsstpv pfssstfttt dsstipalhe itsssatpyr vdtslgtess ttegrlvmv
10381 tldtssqpgr tssspildtr mtesvelgtv tsayqvpsls trltrtdgim ehitkipne
10441 ahrgtirpvk gpqtstspas pkglhtggtk rmettttalk ttttalktts ratlttsvy
```

FIGURE 9 (page 4 of 6)

```
10501 ptlgtltpln asmqmastip temmittpyv fpdvpettss latslgaets talprttps
10561 fnresettas lvsrsgaers pviqtldvss sepdttaswv ihpaetiptv skttpnffh
10621 eldtvsstat shgadvssai ptnispseld altplvtisg tdtsttfptl tksphetet
10681 ttwlthpaet sstiprtipn fshhesdatp siatspgaet ssaipimtvs pgaedlvts
10741 vtssgtdrnm tiptltlspg epktiaslvt hpeaqtssai ptstispavs rlvtsmvts
10801 aaktsttnra ltnspgepat tvslvthpaq tsptvpwtts iffhsksdtt psmttshga
10861 sssavptptv stevpgvvtp lvtssravis ttipiltlsp gepettpsma tshgeeass
10921 iptptvspgv pgvvtslvts sravtsttip iltfslgepe ttpsmatshg teagsavpt
10981 lpevpgmvts lvassravts ttlptltlsp gepettpsma tshgaeasst vptvspevp
11041 vvtslvtsss gvnstsiptl ilspgelett psmatshgae assavptptv spgvsgvvt
11101 lvtssravts ttipiltlss sepettpsma tshgveassa vltvspevpg mvtslvtss
11161 avtsttiptl tissdepett tslvthseak misaiptlav sptvqglvts lvtssgset
11221 afsnltvass qpetidswva hpgteassvv ptltvstgep ftnislvthp aessstlpr
11281 tsrfshseld tmpstvtspe aesssaistt ispgipgvlt slvtssgrdi satfptvpe
11341 pheseatasw vthpavtstt vprttpnysh sepdttpsia tspgaeatsd fptitvspd
11401 pdmvtsqvts sgtdtsitip tltlssgepe tttsfityse thtssaiptl pvspgaskm
11461 tslvissgtd stttfptlte tpyepettai qlihpaetnt mvprttpkfs hsksdttlp
11521 aitspgpeas savstttisp dmsdlvtslv pssgtdtstt fptlsetpye pettatwlt
11581 paetsttvsg tipnfshrgs dtapsmvtsp gvdtrsgvpt ttippsipgv vtsqvtssa
11641 dtstaiptlt pspgepetta ssathpgtqt gftvpirtvp ssepdtmasw vthppqtst
11701 vsrttssfsh sspdatpvma tsprteassa vlttispgap emvtsqitss gaatsttvp
11761 lthspgmpet tallsthprt etsktfpast vfpqvsetta sltirpgaet stalptqtt
11821 slftllvtgt srvdlsptas pgvsaktapl sthpgtetst miptstlslg llettglla
11881 sssaetstst ltltvspavs glssasittd kpqtvtswnt etspsvtsvg ppefsrtvt
11941 ttmtlipsem ptppktshge gvspttilrt tmveatnlat tgssptvakt tttfntlag
12001 lftplttpgm stlasesvts rtsynhrswi sttssynrry wtpatstpvt stfspgist
12061 sipsstaatv pfmvpftlnf titnlqyeed mrhpgsrkfn aterelqgll kplfrnssl
12121 ylysgcrlas lrpekdssat avdaicthrp dpedlgldre rlywelsnlt ngiqelgpy
12181 ldrnslyvng fthrssmptt stpgtstvdv gtsgtpsssp spttagpllm pftlnftit
12241 lqyeedmrrt gsrkfntmes vlqgllkplf kntsvgplys gcrltllrpe kdgaatgvd
12301 icthrldpks pglnreqlyw elskltndie elgpytldrn slyvngfthq ssvsttstp
12361 tstvdlrtsg tpsslsspti maagpllvpf tlnftitnlq ygedmghpgs rkfntterv
12421 qgllgpifkn tsvgplysgc rltslrsekd gaatgvdaic ihhldpkspg lnrerlywe
12481 sqltngikel gpytldrnsl yvngfthrts vptsstpgts tvdlgtsgtp fslpspata
12541 pllvlftlnf titnlkyeed mhrpgsrkfn ttervlqtll gpmfkntsvg llysgcrlt
12601 lrsekdgaat gvdaicthrl dpkspgvdre qlywelsqlt ngikelgpyt ldrnslyvn
12661 fthwipvpts stpgtstvdl gsgtpsslps pttagpllvp ftlnftitnl kyeedmhcp
12721 srkfntterv lqsllgpmfk ntsvgplysg crltllrsek dgaatgvdai cthrldpks
12781 gvdreqlywe lsqltngike lgpytldrns lyvngfthqt sapntstpgt stvdlgtsg
12841 psslpsptsa gpllvpftln ftitnlqyee dmhhpgsrkf nttervlqgl lgpmfknts
12901 gllysgcrlt llrpekngaa tgmdaicshr ldpkspglnr eqlywelsql thgikelgp
12961 tldrnslyvn gfthrssvap tstpgtstvd lgtsgtpssl pspttavpll vpftlnfti
13021 nlqygedmrh pgsrkfntte rvlqgllgpl fknssvgply sgcrlislrs ekdgaatgv
13081 aicthhlnpq spgldreqly wqlsqmtngi kelgpytldr nslyvngfth rssglttst
```

FIGURE 9 (page 5 of 6)

13141 wtstvdlgts gtpspvpspt ttgpllvpft lnftitnlqy eenmghpgsr kfnitesvl
13201 gllkplfkst svgplysgcr ltllrpekdg vatrvdaict hrpdpkipgl drqqlywel
13261 qlthsitelg pytldrdsly vngftqrssv pttstpgtft vqpetsetps slpgptatg
13321 vllpftlnft itnlqyeedm rrpgsrkfnt tervlqgllm plfkntsvss lysgcrltl
13381 rpekdgaatr vdavcthrpd pkspgldrer lywklsqlth gitelgpytl drhslyvng
13441 thqssmtttr tpdtstmhla tsrtpaslsg pmtaspllvl ftinftitnl ryeenmhhp
13501 srkfntterv lqgllrpvfk ntsvgplysg crltllrpkk dgaatkvdai ctyrpdpks
13561 gldreqlywe lsqlthsite lgpytldrds lyvngftqrs svpttsipgt ptvdlgtsg
13621 pvskpgpsaa spllvlftln ftitnlryee nmqhpgsrkf nttervlqgl lrslfksts
13681 gplysgcrlt llrpekdgta tgvdaicthh pdpksprldr eqlywelsql thnitelgp
13741 aldndslfvn gfthrssvst tstpgtptvy lgasktpasi fgpsaashll ilftlnfti
13801 nlryeenmwp gsrkfntter vlqgllrplf kntsvgplys gcrltllrpe kdgeatgvd
13861 icthrpdptg pgldreqlyl elsqlthsit elgpytldrd slyvngfthr ssvpttstg
13921 vseepftlnf tinnlrymad mgqpgslkfn itdnvmqhll splfqrsslg arytgcrvi
13981 lrsvkngaet rvdllctylq plsgpglpik qvfhelsqgt hgitrlgpys ldkdslyln
14041 ynepgpdepp ttpkpattfl pplseattam gyhlktltln ftisnlqysp dmgkgsatf
14101 stegvlqhll rplfqkssmg pfylgcqlis lrpekdgaat gvdttctyhp dpvgpgldi
14161 qlywelsqlt hgvtqlgfyv ldrdslfing yapqnlsirg eyqinfhivn wnlsnpdpt
14221 seyitllrdi qdkvttlykg sqlhdtfrfc lvtnltmdsv lvtvkalfss nldpslveq
14281 fldktlnasf hwlgstyqlv dihvtemess vyqptsssst qhfylnftit nlpysqdka
14341 pgttnyqrnk rniedalnql frnssiksyf sdcqvstfrs vpnrhhtgvd slcnfspla
14401 rvdrvaiyee flrmtrngtq lqnftldrss vlvdgyspnr nepltgnsdl pfwavilig
14461 agllgvitcl icgvlvttrr rkkegeynvq qqcpgyyqsh ldledlq

(B) Peptide 1
14394 14410
nfsplar rvdrvaiyee (SEQ ID NO:01)

(C) Peptide 2
14425 14442
tldrss vlvdgyspnr ne (SEQ ID NO:02)

(D) Peptide 3
14472 14492
cgvlvttrr rkkegeynvq qq (SEQ ID NO:03)

(E) Transmembrane Region:
14452 14475
fwaviligl agllgvitcl icgvl (SEQ ID NO:14)

(F) Peptide containing the cysteine loop peptide:
14367 14398
ksyf sdcqvstfrs vpnrhhtgvd slcnfspl (SEQ ID NO:15)

FIGURE 9 (page 6 of 6)

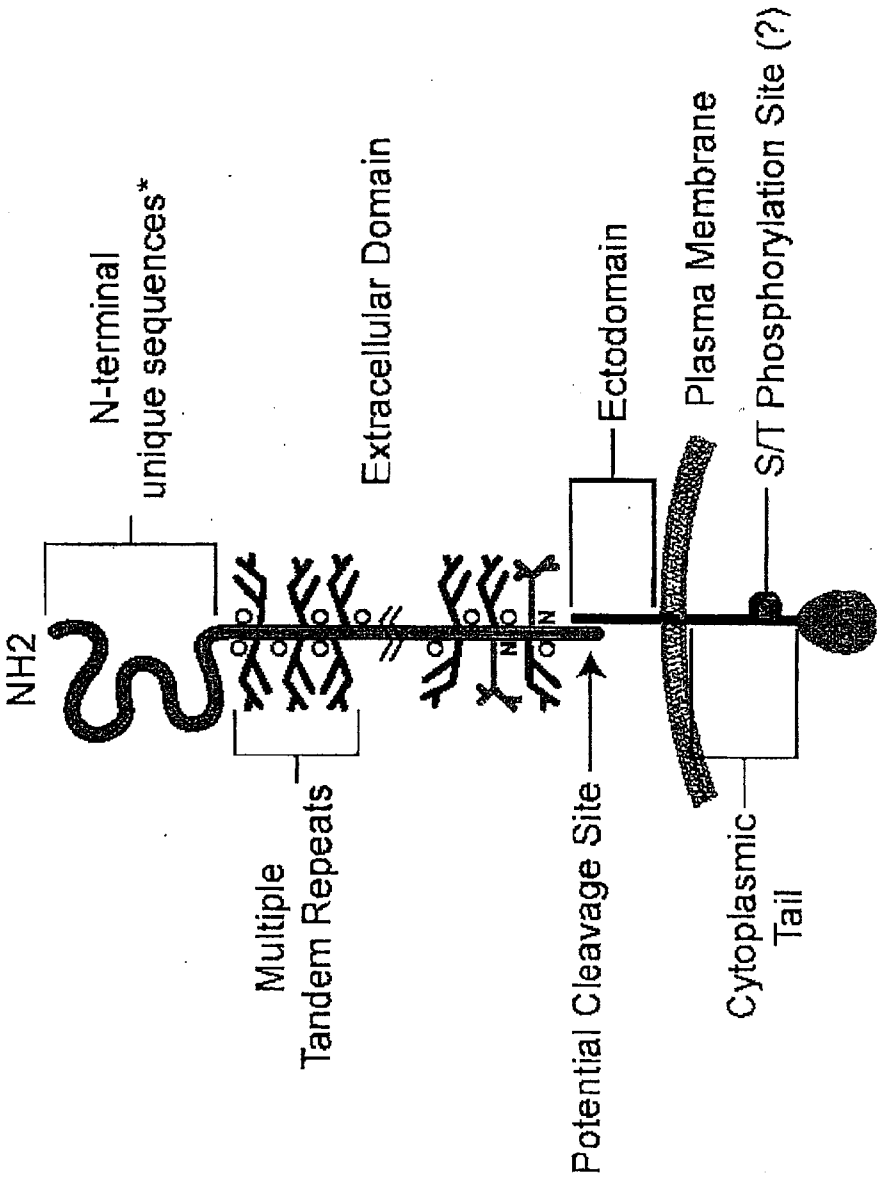

NH2
N-terminal
unique sequences*
Extracellular Domain
Ectodomain
Plasma Membrane
S/T Phosphorylation Site (?)
Multiple
Tandem Repeats
Potential Cleavage Site
Cytoplasmic
Tail

Figure 10

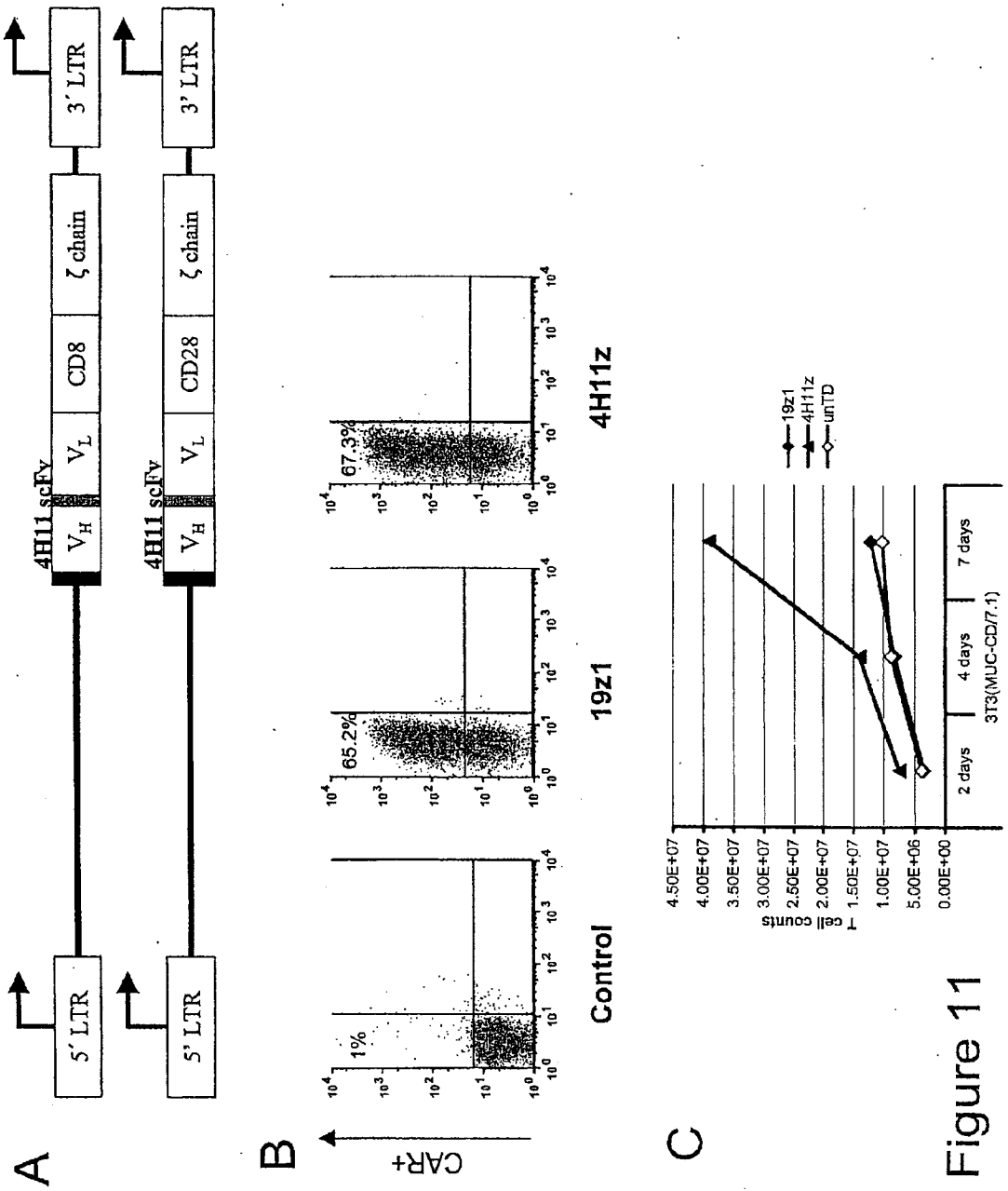

A
5' LTR
3' LTR
4H11 scFv
V_H
V_L
CD8
CD28
ζ chain
B
CAR+
Control
19z1
4H11z
1%
65.2%
67.3%
C
19z1
4H11z
unTD
cell counts
4.50E+07
4.00E+07
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
2 days
4 days
7 days
3T3(MUC-CD/7.1)

Figure 11

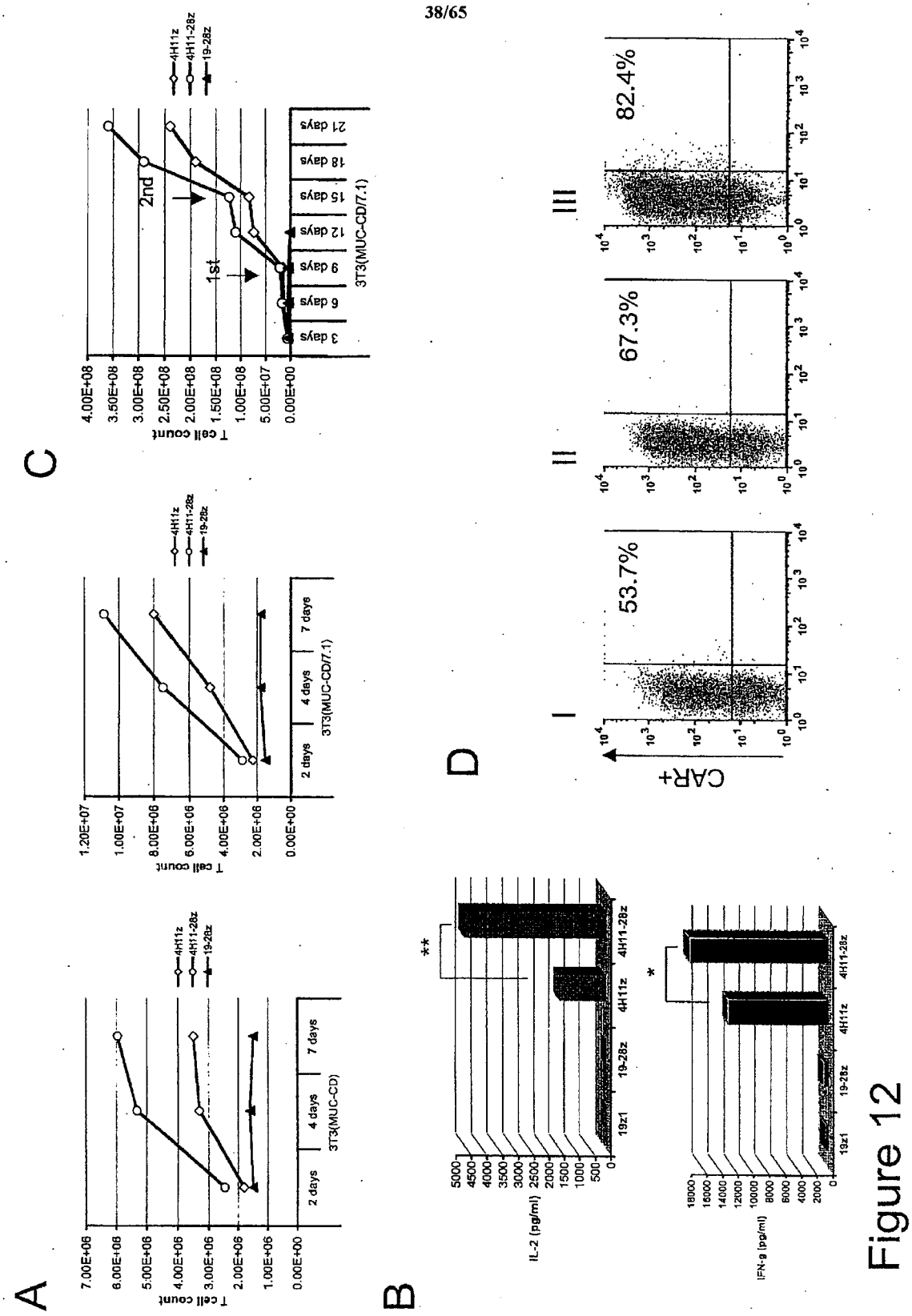

A
T cell count
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
4H11z
4H11-28z
19-28z
2 days
4 days
7 days
3T3(MUC-CD)
B
IL-2 (pg/ml)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
19z1
19-28z
4H11z
4H11-28z
IFN-g (pg/ml)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
C
T cell count
4.00E+08
3.50E+08
3.00E+08
2.50E+08
2.00E+08
1.50E+08
1.00E+08
5.00E+07
0.00E+00
1st
2nd
3 days
6 days
9 days
12 days
15 days
18 days
21 days
3T3(MUC-CD/7.1)
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
2 days
4 days
7 days
3T3(MUC-CD/7.1)
D
I
II
III
CAR+
53.7%
67.3%
82.4%

Figure 12

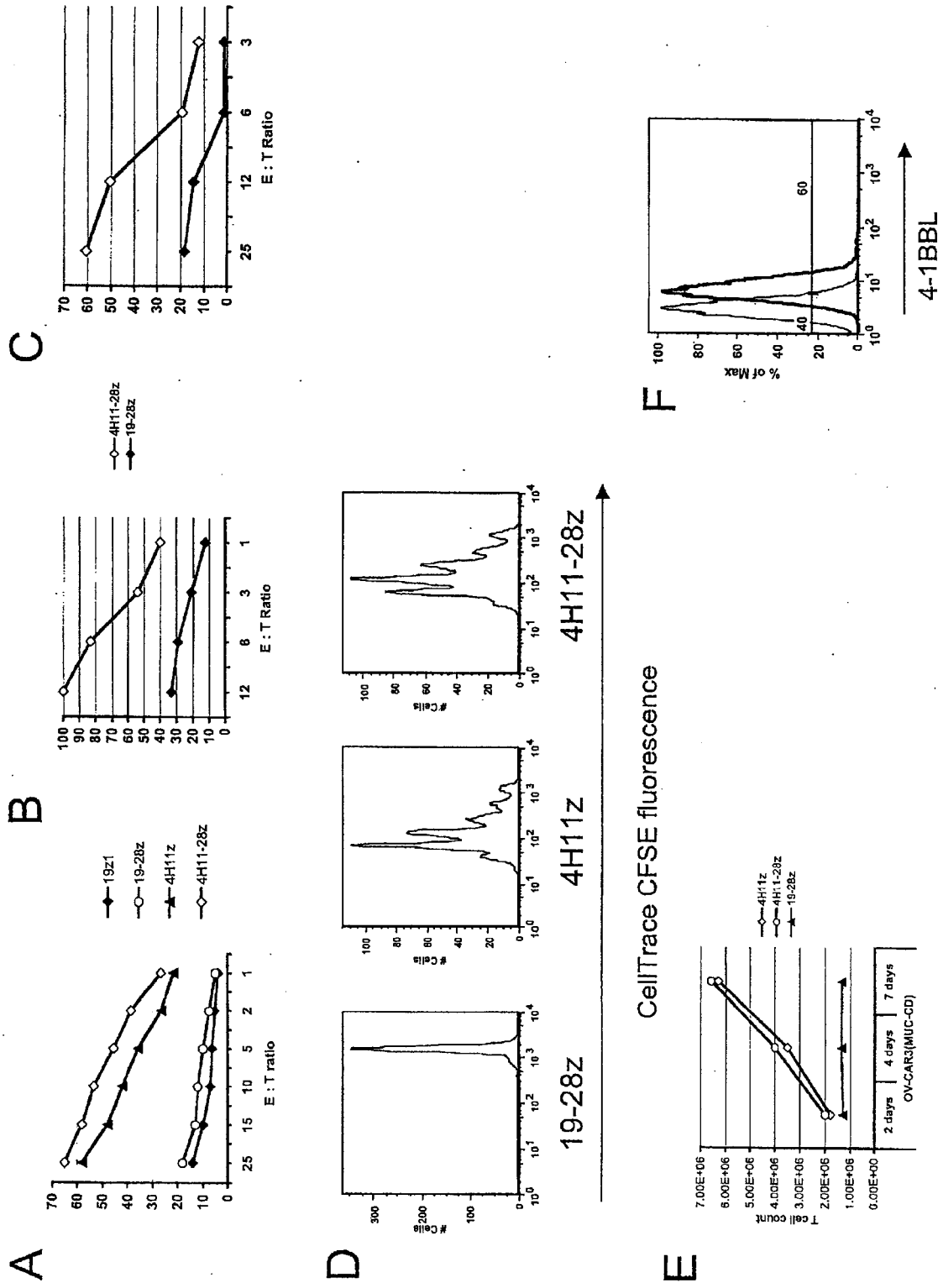
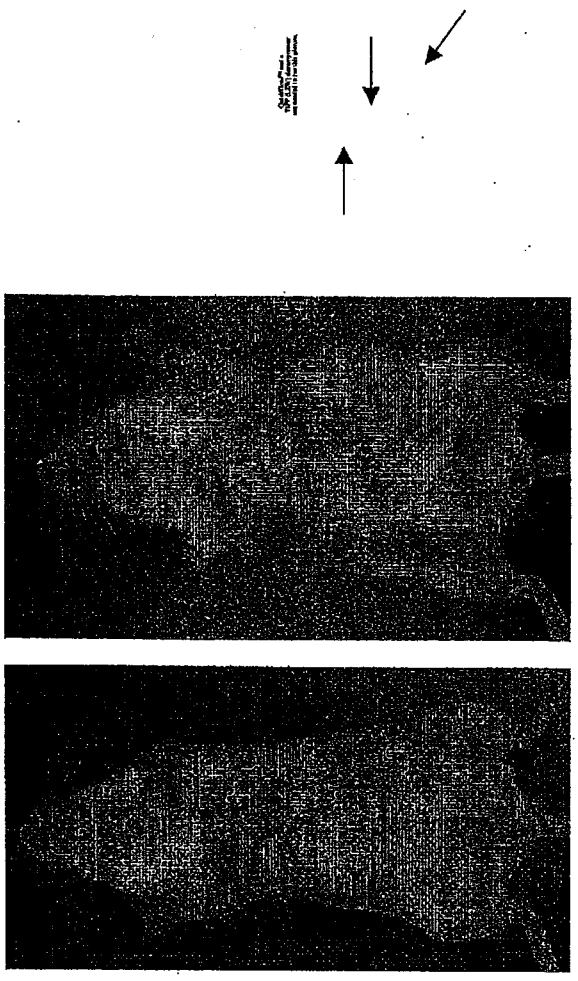
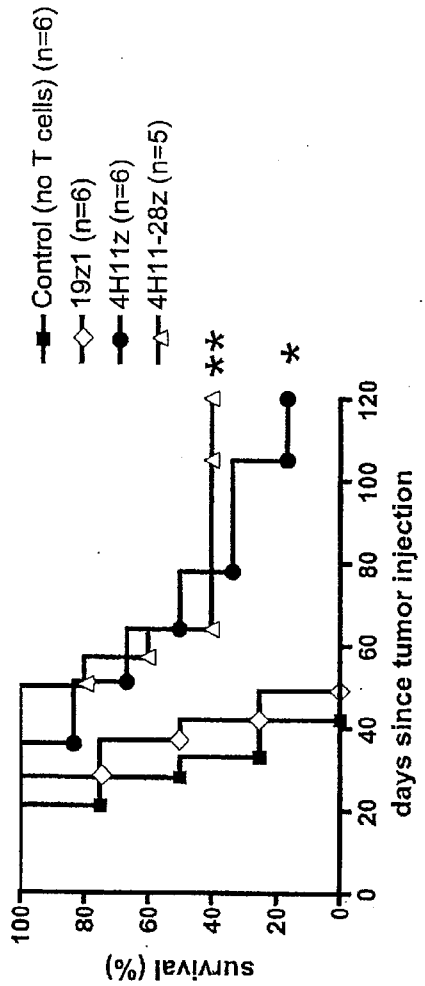

A
B
C
D
E
F
E : T ratio
19z1
19-28z
4H11z
4H11-28z
4H11-28z
19-28z
E : T Ratio
4H11-28z
19-28z
E : T Ratio
Cells
19-28z
4H11z
4H11-28z
CellTrace CFSE fluorescence
T cell count
4H11z
4H11-28z
19-28z
2 days
4 days
7 days
OV-CAR3(MUC-CD)
% of Max
4-1BBL

Figure 13



A


Control (no T cells) (n=6)
19z1 (n=6)
4H11z (n=6)
4H11-28z (n=5)
**
*
survival (%)
days since tumor injection
0
20
40
60
80
100
120

B

Figure 14

A

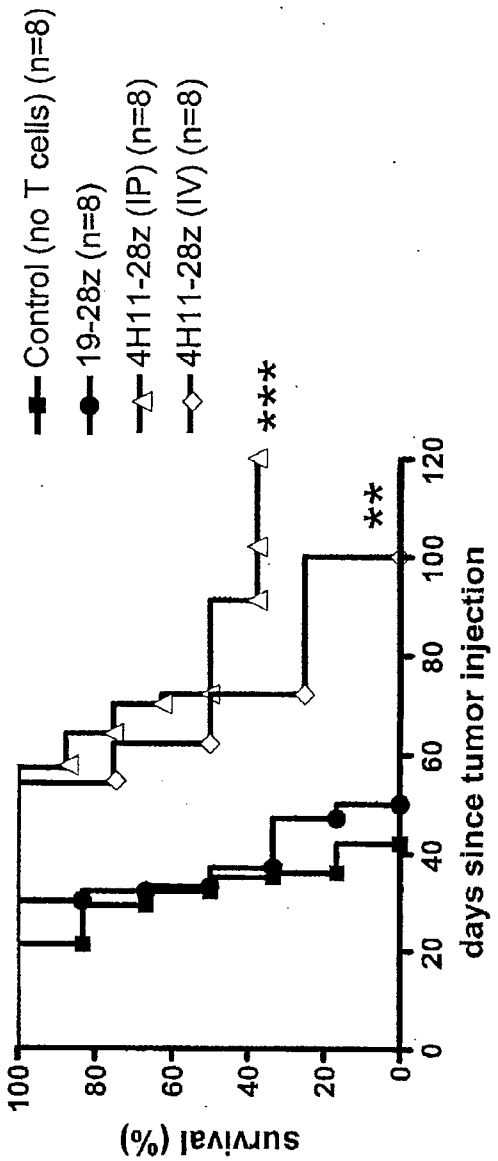


Figure 15 (1 of 3)

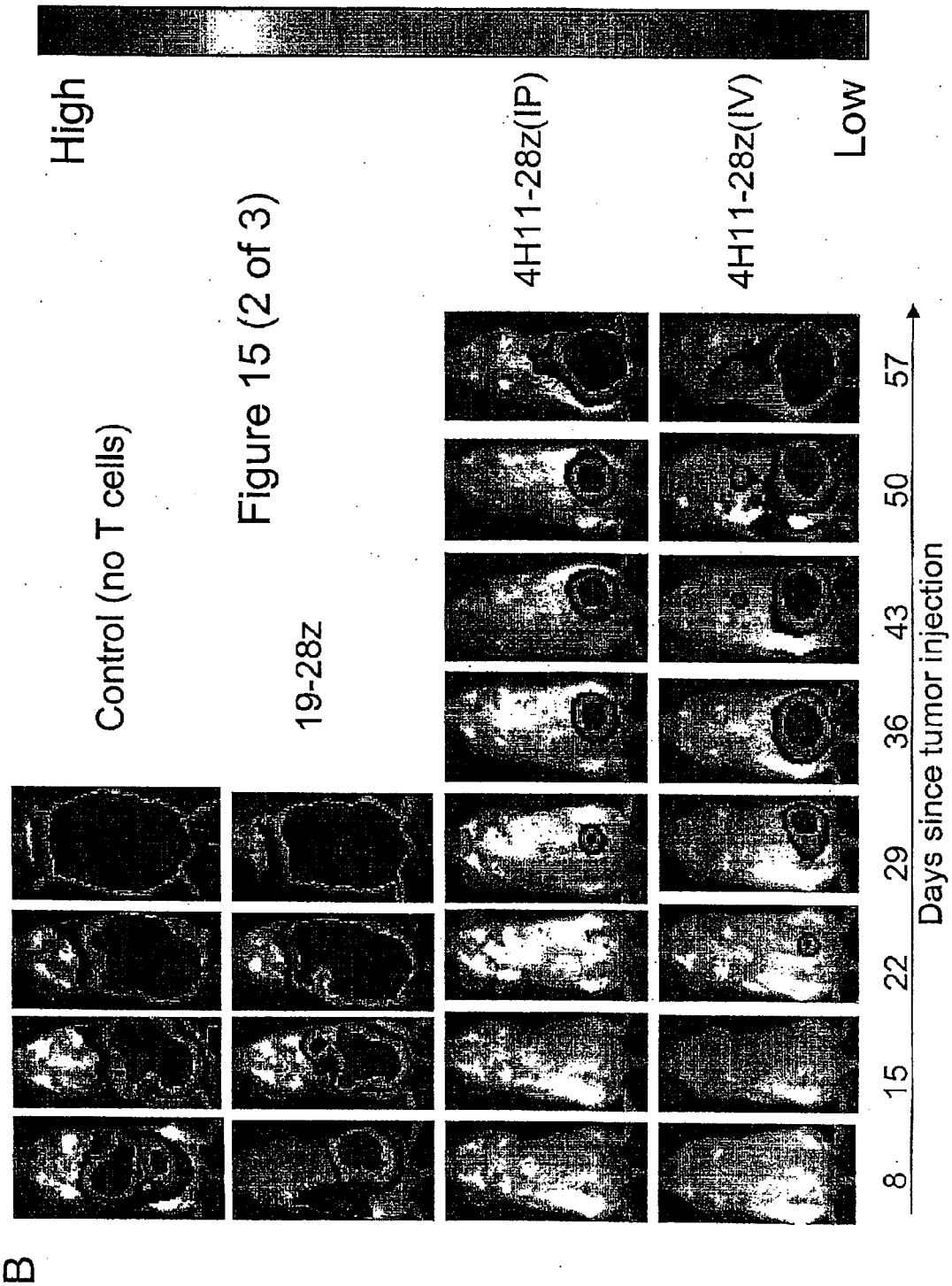


Figure 15 (2 of 3)

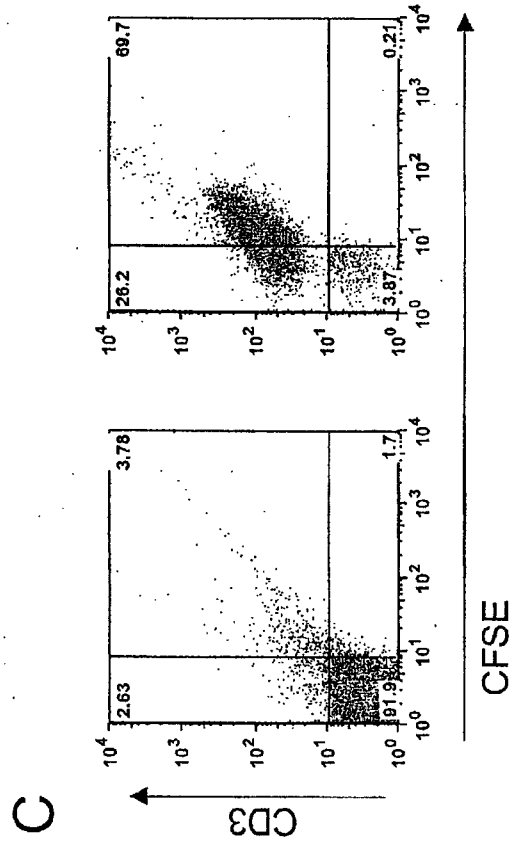


Figure 15 (3 of 3)

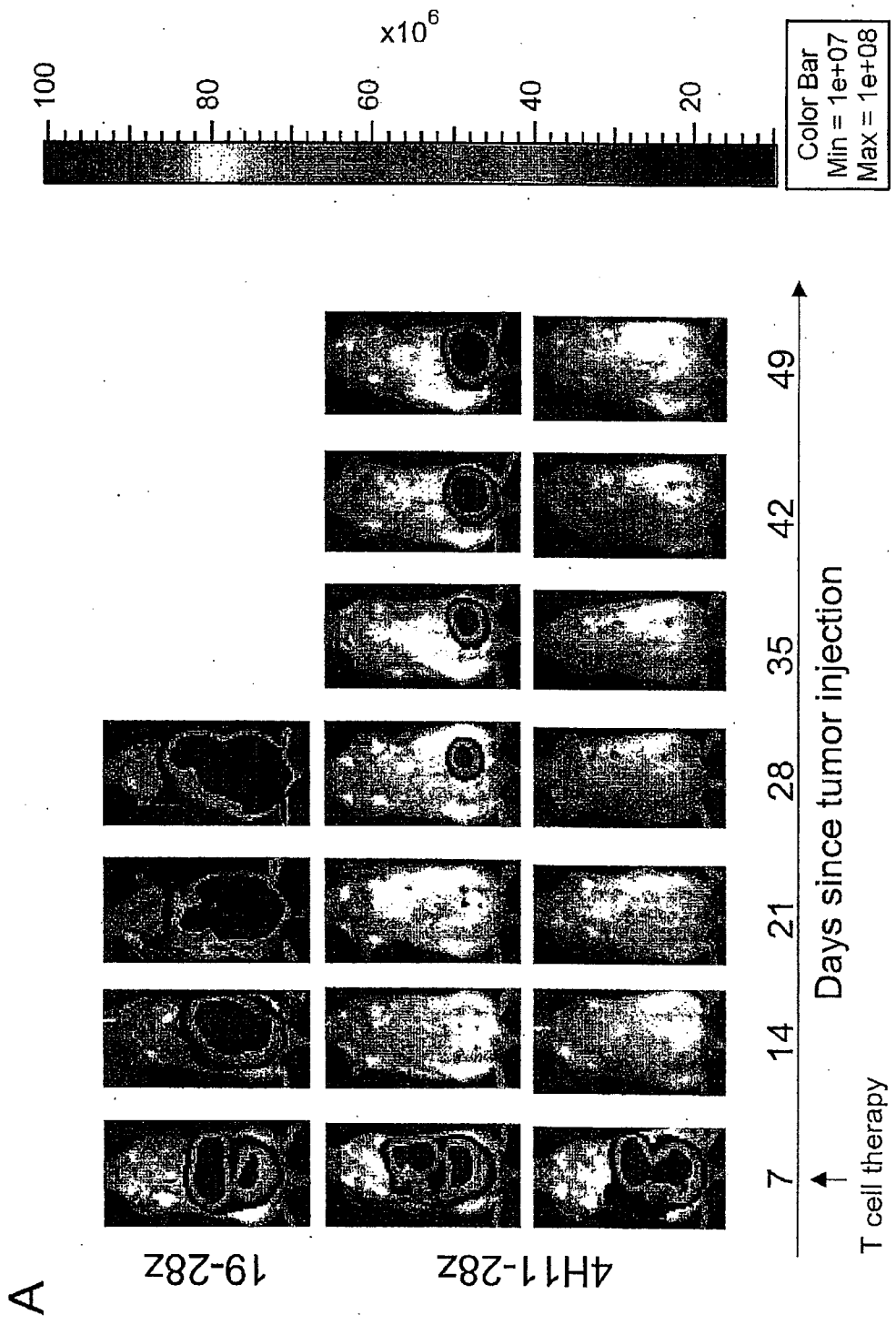


Figure 16 (1 of 2)

B

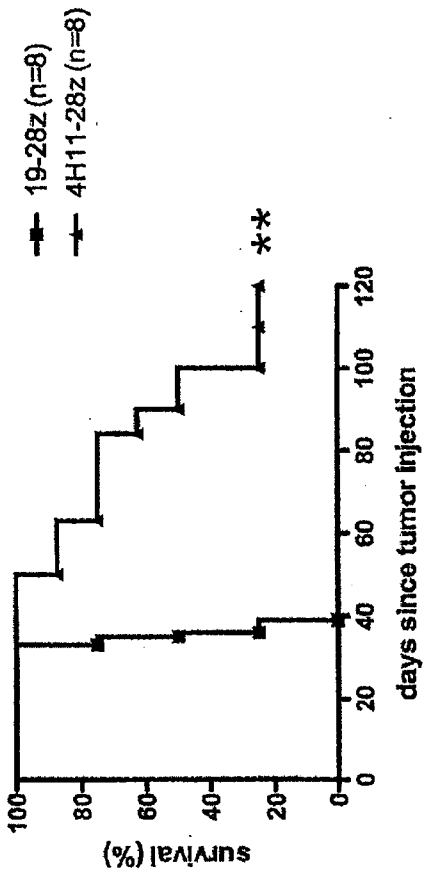


Figure 16 (2 of 2)

CD8 leader sequence
ATGGCTC TCCCAGTGAC TGCCCTACTG CTTCCCCTAG CGCTTCTCCT GCATGCAGAG (SEQ ID NO:32)

CD3 zeta chain intracellular domain
AGAGT GAAGTTCAGC AGGAGCGCAG AGCCCCCCGC GTACCAGCAG GGCCAGAACC AGCTCTATAA CGAGCTCAAT CTAGGACGAA GAGAGGAGTA CGATGTTTTG GACAAGAGAC GTGGCCGGGA CCCTGAGATG GGGGGAAAGC CGAGAAGGAA GAACCCTCAG GAAGGCCTGT ACAATGAACT GCAGAAAGAT AAGATGGCGG AGGCCTACAG TGAGATTGGG ATGAAAGGCG AGCGCCGGAG GGGCAAGGGG CACGATGGCC TTTACCAGGG TCTCAGTACA GCCACCAAGG ACACCTACGA CGCCCTTCAC ATGCAGGCCC TGCCCCCTCG (SEQ ID NO:33)

(G4S)3 serine-glycine linker
GGTG GAGGTGGATC AGGTGGAGGT GGATCTGGTGGAGGTGGATC T (SEQ ID NO:34)

CD8 transmembrane domain
GCGGCCGCAC CCACCACGAC GCCAGCGCCG CGACCACCAA CCCCGGCGCC CACGATCGCG TCGCAGCCCC TGTCCCTGCG CCCAGAGGCG TGCCGGCCAG CGGCGGGGGG CGCAGTGCAC ACGAGGGGGC TGGACTTCGC CTGTGATATCTACATCTGGG CGCCCTTGGC CGGGACTTGT GGGGTCCTTC TCCTGTCACT GGTTATCACC CTTTACTGCA ACCAC (SEQ ID NO:35)

CD28 transmembrane + intracellular domains (-STOP)
CAA TTGAAGTTAT GTATCCTCCT CCTTACCTAG ACAATGAGAA GAGCAATGGA ACCATTATCC ATGTGAAAGG GAAACACCTT TGTCCAAGTC CCCTATTTCC CGGACCTTCT AAGCCCTTTT GGGTGCTGGT GGTGGTTGGT GGAGTCCTGG CTTGCTATAG CTTGCTAGTA ACAGTGGCCT TTATTATTTT CTGGGTGAGG AGTAAGAGGA GCAGGCTCCT (SEQ ID NO:36)

FIGURE 17

```
          BamHI
          ------
     1    GGATCCGGAT TAGTCCAATT TGTTAAAGAC AGGATATCAG TGGTCCAGGC TCTAGTTTTG ACTCAACAAT ATCACCAGCT GAAGCCTATA GAGTACGAGC
          CCTAGGCCTA ATCAGGTTAA ACAATTTCTG TCCTATAGTC ACCAGGTCCG AGATCAAAAC TGAGTTGTTA TAGTGGTCGA CTTCGGATAT CTCATGCTCG
   101    CATAGATAAA ATAAAAGATT TTATTTAGTC TCCAGAAAAA GGGGGGAATG AAAGACCCCA CCTGTAGGTT TGGCAAGCTA GCTTAAGTAA CGCCATTTTG
          GTATCTATTT TATTTTCTAA AATAAATCAG AGGTCTTTTT CCCCCCTTAC TTTCTGGGGT GGACATCCAA ACCGTTCGAT CGAATTCATT GCGGTAAAAC
   201    CAAGGCATGG AAAAATACAT AACTGAGAAT AGAGAAGTTC AGATCAAGGT CAGGAACAGA TGGAACAGCT GAATATGGGC CAAACAGGAT ATCTGTGGTA
          GTTCCGTACC TTTTTATGTA TTGACTCTTA TCTCTTCAAG TCTAGTTCCA GTCCTTGTCT ACCTTGTCGA CTTATACCCG GTTTGTCCTA TAGACACCAT
   301    AGCAGTTCCT GCCCCGGCTC AGGGCCAAGA ACAGATGGAA CAGCTGAATA TGGGCCAAAC AGGATATCTG TGGTAAGCAG TTCCTGCCCC GGCTCAGGGC
          TCGTCAAGGA CGGGGCCGAG TCCCGGTTCT TGTCTACCTT GTCGACTTAT ACCCGGTTTG TCCTATAGAC ACCATTCGTC AAGGACGGGG CCGAGTCCCG
   401    CAAGAACAGA TGGTCCCCAG ATGCGGTCCA GCCCTCAGCA GTTTCTAGAG AACCATCAGA TGTTTCCAGG GTGCCCCAAG GACCTGAAAT GACCCTGTGC
          GTTCTTGTCT ACCAGGGGTC TACGCCAGGT CGGGAGTCGT CAAAGATCTC TTGGTAGTCT ACAAAGGTCC CACGGGGTTC CTGGACTTTA CTGGGACACG
   501    CTTATTTGAA CTAACCAATC AGTTCGCTTC TCGCTTCTGT TCGCGCGCTT CTGCTCCCCG AGCTCAATAA AAGAGCCCAC AACCCCTCAC TCGGGGCGCC
          GAATAAACTT GATTGGTTAG TCAAGCGAAG AGCGAAGACA AGCGCGCGAA GACGAGGGGC TCGAGTTATT TTCTCGGGTG TTGGGGAGTG AGCCCCGCGG
   601    AGTCCTCCGA TTGACTGAGT CGCCCGGGTA CCCGTGTATC CAATAAACCC TCTTGCAGTT GCATCCGACT TGTGGTCTCG CTGTTCCTTG GGAGGGTCTC
          TCAGGAGGCT AACTGACTCA GCGGGCCCAT GGGCACATAG GTTATTTGGG AGAACGTCAA CGTAGGCTGA ACACCAGAGC GACAAGGAAC CCTCCCAGAG
   701    CTCTGAGTGA TTGACTACCC GTCAGCGGGG GTCTTTCACA CATGCAGCAT GTATCAAAAT TAATTTGGTT TTTTTTCTTA AGTATTTACA TTAAATGGCC
          GAGACTCACT AACTGATGGG CAGTCGCCCC CAGAAAGTGT GTACGTCGTA CATAGTTTTA ATTAAACCAA AAAAAAGAAT TCATAAATGT AATTTACCGG
   801    ATAGTACTTA AAGTTACATT GGCTTCCTTG AAATAAACAT GGAGTATTCA GAATGTGTCA TAAATATTTC TAATTTTAAG ATAGTATCTC CATTGGCTTT
          TATCATGAAT TTCAATGTAA CCGAAGGAAC TTTATTTGTA CCTCATAAGT CTTACACAGT ATTTATAAAG ATTAAAATTC TATCATAGAG GTAACCGAAA
   901    CTACTTTTTC TTTTATTTTT TTTTGTCCTC TGTCTTCCAT TTGTTGTTGT TGTTGTTTGT TTGTTTGTTT GTTGGTTGGT TGGTTAATTT TTTTTAAAG
          GATGAAAAAG AAAATAAAAA AAAACAGGAG ACAGAAGGTA AACAACAACA ACAACAAACA AACAAACAAA CAACCAACCA ACCAATTAAA AAAAAATTTC
  1001    ATCCTACACT ATAGTTCAAG CTAGACTATT AGCTACTCTG TAACCCAGGG TGACCTTGAA GTCATGGGTA GCCTGCTGTT TTAGCCTTCC CACATCTAAG
          TAGGATGTGA TATCAAGTTC GATCTGATAA TCGATGAGAC ATTGGGTCCC ACTGGAACTT CAGTACCCAT CGGACGACAA AATCGGAAGG GTGTAGATTC
  1101    ATTACAGGTA TGAGCTATCA TTTTTGGTAT ATTGATTGAT TGATTGATTG ATGTGTGTGT GTGTGATTGT GTTTGTGTGT GTGACTGTGA AAATGTGTGT
          TAATGTCCAT ACTCGATAGT AAAAACCATA TAACTAACTA ACTAACTAAC TACACACACA CACACTAACA CAAACACACA CACTGACACT TTTACACACA
  1201    ATGGGTGTGT GTGAATGTGT GTATGTATGT GTGTGTGTGA GTGTGTGTGT GTGTGTGTGC ATGTGTGTGT GTGTGACTGT GTCTATGTGT ATGACTGTGT
          TACCCACACA CACTTACACA CATACATACA CACACACACT CACACACACA CACACACACG TACACACACA CACACTGACA CAGATACACA TACTGACACA
  1301    GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTTGTGAAAA AATATTCTAT GGTAGTGAGA GCCAACGCTC CGGCTCAGGT GTCAGGTTGG
          CACACACACA CACACACACA CACACACACA CACACACACA CAACACTTTT TTATAAGATA CCATCACTCT CGGTTGCGAG GCCGAGTCCA CAGTCCAACC
                                          EcoRI
                                          ------
  1401    TTTTTGAGAC AGAGTCTTTC ACTTAGCTTG GAATTCACTG GCCGTCGTTT TACAACGTCG TGACTGGGAA AACCCTGGCG TTACCCAACT TAATCGCCTT
          AAAAACTCTG TCTCAGAAAG TGAATCGAAC CTTAAGTGAC CGGCAGCAAA ATGTTGCAGC ACTGACCCTT TTGGGACCGC AATGGGTTGA ATTAGCGGAA
  1501    GCAGCACATC CCCCTTTCGC CAGCTGGCGT AATAGCGAAG AGGCCCGCAC CGATCGCCCT TCCCAACAGT TGCGCAGCCT GAATGGCGAA TGGCGCCTGA
          CGTCGTGTAG GGGGAAAGCG GTCGACCGCA TTATCGCTTC TCCGGGCGTG GCTAGCGGGA AGGGTTGTCA ACGCGTCGGA CTTACCGCTT ACCGCGGACT
  1601    TGCGGTATTT TCTCCTTACG CATCTGTGCG GTATTTCACA CCGCATATGG TGCACTCTCA GTACAATCTG CTCTGATGCC GCATAGTTAA GCCAGCCCCG
          ACGCCATAAA AGAGGAATGC GTAGACACGC CATAAAGTGT GGCGTATACC ACGTGAGAGT CATGTTAGAC GAGACTACGG CGTATCAATT CGGTCGGGGC
  1701    ACACCCGCCA ACACCCGCTG ACGCGCCCTG ACGGGCTTGT CTGCTCCCGG CATCCGCTTA CAGACAAGCT GTGACCGTCT CCGGGAGCTG CATGTGTCAG
          TGTGGGCGGT TGTGGGCGAC TGCGCGGGAC TGCCCGAACA GACGAGGGCC GTAGGCGAAT GTCTGTTCGA CACTGGCAGA GGCCCTCGAC GTACACAGTC
  1801    AGGTTTTCAC CGTCATCACC GAAACGCGCG ATGACGAAAG GGCCTCGTGA TACGCCTATT TTTATAGGTT AATGTCATGA TAATAATGGT TTCTTAGACG
          TCCAAAAAGTG GCAGTAGTGG CTTTGCGCGC TACTGCTTTC CCGGAGCACT ATGCGGATAA AAATATCCAA TTACAGTACT ATTATTACCA AAGAATCTGC
  1901    TCAGGTGGCA CTTTTCGGGG AAATGTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCTGAT
          AGTCCACCGT GAAAAGCCCC TTTACACGCG CCTTGGGGAT AAACAAATAA AAAGATTTAT GTAAGTTTAT ACATAGGCGA GTACTCTGTT ATTGGGACTA
  2001    AAATGCTTCA ATAATATTGA AAAAGGAAGA GTATGAGTAT TCAACATTTC CGTGTCGCCC TTATTCCCTT TTTTGCGGCA TTTTGCCTTC CTGTTTTTGC
          TTTACGAAGT TATTATAACT TTTTCCTTCT CATACTCATA AGTTGTAAAG GCACAGCGGG AATAAGGGAA AAAACGCCGT AAAACGGAAG GACAAAAACG
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2101	TCACCCAGAA	ACGCTGGTGA	AAGTAAAAGA	TGCTGAAGAT	CAGTTGGGTG	CACGAGTGGG	TTACATCGAA	CTGGATCTCA	ACAGCGGTAA	GATCCTTGAG
	AGTGGGTCTT	TGCGACCACT	TTCATTTTCT	ACGACTTCTA	GTCAACCCAC	GTGCTCACCC	AATGTAGCTT	GACCTAGAGT	TGTCGCCATT	CTAGGAACTC
2201	AGTTTTCGCC	CCGAAGAACG	TTTTCCAATG	ATGAGCACTT	TTAAAGTTCT	GCTATGTGGC	GCGGTATTAT	CCCGTATTGA	CGCCGGGCAA	GAGCAACTCG
	TCAAAAGCGG	GGCTTCTTGC	AAAAGGTTAC	TACTCGTGAA	AATTTCAAGA	CGATACACCG	CGCCATAATA	GGGCATAACT	GCGGCCCGTT	CTCGTTGAGC
2301	GTCGCCGCAT	ACACTATTCT	CAGAATGACT	TGGTTGAGTA	CTCACCAGTC	ACAGAAAAGC	ATCTTACGGA	TGGCATGACA	GTAAGAGAAT	TATGCAGTGC
	CAGCGGCGTA	TGTGATAAGA	GTCTTACTGA	ACCAACTCAT	GAGTGGTCAG	TGTCTTTTCG	TAGAATGCCT	ACCGTACTGT	CATTCTCTTA	ATACGTCACG
2401	TGCCATAACC	ATGAGTGATA	ACACTGCGGC	CAACTTACTT	CTGACAACGA	TCGGAGGACC	GAAGGAGCTA	ACCGCTTTTT	TGCACAACAT	GGGGGATCAT
	ACGGTATTGG	TACTCACTAT	TGTGACGCCG	GTTGAATGAA	GACTGTTGCT	AGCCTCCTGG	CTTCCTCGAT	TGGCGAAAAA	ACGTGTTGTA	CCCCCTAGTA
2501	GTAACTCGCC	TTGATCGTTG	GGAACCGGAG	CTGAATGAAG	CCATACCAAA	CGACGAGCGT	GACACCACGA	TGCCTGTAGC	AATGGCAACA	ACGTTGCGCA
	CATTGAGCGG	AACTAGCAAC	CCTTGGCCTC	GACTTACTTC	GGTATGGTTT	GCTGCTCGCA	CTGTGGTGCT	ACGGACATCG	TTACCGTTGT	TGCAACGCGT
2601	AACTATTAAC	TGGCGAACTA	CTTACTCTAG	CTTCCCGGCA	ACAATTAATA	GACTGGATGG	AGGCGGATAA	AGTTGCAGGA	CCACTTCTGC	GCTCGGCCCT
	TTGATAATTG	ACCGCTTGAT	GAATGAGATC	GAAGGGCCGT	TGTTAATTAT	CTGACCTACC	TCCGCCTATT	TCAACGTCCT	GGTGAAGACG	CGAGCCGGGA
2701	TCCGGCTGGC	TGGTTTATTG	CTGATAAATC	TGGAGCCGGT	GAGCGTGGGT	CTCGCGGTAT	CATTGCAGCA	CTGGGGCCAG	ATGGTAAGCC	CTCCCGTATC
	AGGCCGACCG	ACCAAATAAC	GACTATTTAG	ACCTCGGCCA	CTCGCACCCA	GAGCGCCATA	GTAACGTCGT	GACCCCGGTC	TACCATTCGG	GAGGGCATAG
2801	GTAGTTATCT	ACACGACGGG	GAGTCAGGCA	ACTATGGATG	AACGAAATAG	ACAGATCGCT	GAGATAGGTG	CCTCACTGAT	TAAGCATTGG	TAACTGTCAG
	CATCAATAGA	TGTGCTGCCC	CTCAGTCCGT	TGATACCTAC	TTGCTTTATC	TGTCTAGCGA	CTCTATCCAC	GGAGTGACTA	ATTCGTAACC	ATTGACAGTC
2901	ACCAAGTTTA	CTCATATATA	CTTTAGATTG	ATTTAAAACT	TCATTTTTAA	TTTAAAAGGA	TCTAGGTGAA	GATCCTTTTT	GATAATCTCA	TGACCAAAAT
	TGGTTCAAAT	GAGTATATAT	GAAATCTAAC	TAAATTTTGA	AGTAAAAATT	AAATTTTCCT	AGATCCACTT	CTAGGAAAAA	CTATTAGAGT	ACTGGTTTTA
3001	CCCTTAACGT	GAGTTTTCGT	TCCACTGAGC	GTCAGACCCC	GTAGAAAAGA	TCAAAGGATC	TTCTTGAGAT	CCTTTTTTTC	TGCGCGTAAT	CTGCTGCTTG
	GGGAATTGCA	CTCAAAAGCA	AGGTGACTCG	CAGTCTGGGG	CATCTTTTCT	AGTTTCCTAG	AAGAACTCTA	GGAAAAAAAG	ACGCGCATTA	GACGACGAAC
3101	CAAACAAAAA	AACCACCGCT	ACCAGCGGTG	GTTTGTTTGC	CGGATCAAGA	GCTACCAACT	CTTTTTCCGA	AGGTAACTGG	CTTCAGCAGA	GCGCAGATAC
	GTTTGTTTTT	TTGGTGGCGA	TGGTCGCCAC	CAAACAAACG	GCCTAGTTCT	CGATGGTTGA	GAAAAAGGCT	TCCATTGACC	GAAGTCGTCT	CGCGTCTATG
3201	CAAATACTGT	CCTTCTAGTG	TAGCCGTAGT	TAGGCCACCA	CTTCAAGAAC	TCTGTAGCAC	CGCCTACATA	CCTCGCTCTG	CTAATCCTGT	TACCAGTGGC
	GTTTATGACA	GGAAGATCAC	ATCGGCATCA	ATCCGGTGGT	GAAGTTCTTG	AGACATCGTG	GCGGATGTAT	GGAGCGAGAC	GATTAGGACA	ATGGTCACCG
3301	TGCTGCCAGT	GGCGATAAGT	CGTGTCTTAC	CGGGTTGGAC	TCAAGACGAT	AGTTACCGGA	TAAGGCGCAG	CGGTCGGGCT	GAACGGGGGG	TTCGTGCACA
	ACGACGGTCA	CCGCTATTCA	GCACAGAATG	GCCCAACCTG	AGTTCTGCTA	TCAATGGCCT	ATTCCGCGTC	GCCAGCCCGA	CTTGCCCCCC	AAGCACGTGT
3401	CAGCCCAGCT	TGGAGCGAAC	GACCTACACC	GAACTGAGAT	ACCTACAGCG	TGAGCATTGA	GAAAGCGCCA	CGCTTCCCGA	AGGGAGAAAG	GCGGACAGGT
	GTCGGGTCGA	ACCTCGCTTG	CTGGATGTGG	CTTGACTCTA	TGGATGTCGC	ACTCGTAACT	CTTTCGCGGT	GCGAAGGGCT	TCCCTCTTTC	CGCCTGTCCA
3501	ATCCGGTAAG	CGGCAGGGTC	GGAACAGGAG	AGCGCACGAG	GGAGCTTCCA	GGGGGAAACG	CCTGGTATCT	TTATAGTCCT	GTCGGGTTTC	GCCACCTCTG
	TAGGCCATTC	GCCGTCCCAG	CCTTGTCCTC	TCGCGTGCTC	CCTCGAAGGT	CCCCCTTTGC	GGACCATAGA	AATATCAGGA	CAGCCCAAAG	CGGTGGAGAC
3601	ACTTGAGCGT	CGATTTTTGT	GATGCTCGTC	AGGGGGGCGG	AGCCTATGGA	AAAACGCCAG	CAACGCGGCC	TTTTTACGGT	TCCTGGCCTT	TTGCTGGCCT
	TGAACTCGCA	GCTAAAAACA	CTACGAGCAG	TCCCCCCGCC	TCGGATACCT	TTTTGCGGTC	GTTGCGCCGG	AAAAATGCCA	AGGACCGGAA	AACGACCGGA
3701	TTTGCTCACA	TGTTCTTTCC	TGCGTTATCC	CCTGATTCTG	TGGATAACCG	TATTACCGCC	TTTGAGTGAG	CTGATACCGC	TCGCCGCAGC	CGAACGACCG
	AAACGAGTGT	ACAAGAAAGG	ACGCAATAGG	GGACTAAGAC	ACCTATTGGC	ATAATGGCGG	AAACTCACTC	GACTATGGCG	AGCGGCGTCG	GCTTGCTGGC
3801	AGCGCAGCGA	GTCAGTGAGC	GAGGAAGCGG	AAGAGCGCCC	AATACGCAAA	CCGCCTCTCC	CCGCGCGTTG	GCCGATTCAT	TAATGCAGCT	GGCACGACAG
	TCGCGTCGCT	CAGTCACTCG	CTCCTTCGCC	TTCTCGCGGG	TTATGCGTTT	GGCGGAGAGG	GGCGCGCAAC	CGGCTAAGTA	ATTACGTCGA	CCGTGCTGTC
3901	GTTTCCCGAC	TGGAAAGCGG	GCAGTGAGCG	CAACGCAATT	AATGTGAGTT	AGCTCACTCA	TTAGGCACCC	CAGGCTTTAC	ACTTTATGCT	TCCGGCTCGT
	CAAAGGGCTG	ACCTTTCGCC	CGTCACTCGC	GTTGCGTTAA	TTACACTCAA	TCGAGTGAGT	AATCCGTGGG	GTCCGAAATG	TGAAATACGA	AGGCCGAGCA
4001	ATGTTGTGTG	GAATTGTGAG	CGGATAACAA	TTTCACACAG	GAAACAGCTA	TGACCATGAT	TACGCCAAGC	TTTGCTCTTA	GGAGTTTCCT	AATACATCCC
	TACAACACAC	CTTAACACTC	GCCTATTGTT	AAAGTGTGTC	CTTTGTCGAT	ACTGGTACTA	ATGCGGTTCG	AAACGAGAAT	CCTCAAAGGA	TTATGTAGGG
4101	AAACTCAAAT	ATATAAAGCA	TTTGACTTGT	TCTATGCCCT	AGGGGGCGGG	GGAAGCTAA	GCCAGCTTTT	TTTAACATTT	AAAATGTTAA	TTCCATTTTA
	TTTGAGTTTA	TATATTTCGT	AAACTGAACA	AGATACGGGA	TCCCCCGCCC	CCCTTCGATT	CGGTCGAAAA	AAATTGTAAA	TTTTACAATT	AAGGTAAAAT
4201	AATGCACAGA	TGTTTTTATT	TCATAAGGGT	TTCAATGTGC	ATGAATGCTG	CAATATTCCT	GTTACCAAAG	CTAGTATAAA	TAAAAATAGA	TAAACGTGGA
	TTACGTGTCT	ACAAAAATAA	AGTATTCCCA	AAGTTACACG	TACTTACGAC	GTTATAAGGA	CAATGGTTTC	GATCATATTT	ATTTTTATCT	ATTTGCACCT
4301	AATTACTTAG	AGTTTCTGTC	ATTAACGTTT	CCTTCCTCAG	TTGACAACAT	AAATGCGCTG	CTGAGCAAGC	CAGTTTGCAT	CTGTCAGGAT	CAATTTCCCA

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        TTAATGAATC TCAAAGACAG TAATTGCAAA GGAAGGAGTC AACTGTTGTA TTTACGCGAC GACTCGTTCG GTCAAACGTA GACAGTCCTA GTTAAAGGGT
4401    TTATGCCAGT CATATTAATT ACTAGTCAAT TAGTTGATTT TTATTTTTGA CATATACATG TGAATGAAAG ACCCCACCTG TAGGTTTGGC AAGCTAGCTT
        AATACGGTCA GTATAATTAA TGATCAGTTA ATCAACTAAA AATAAAAACT GTATATGTAC ACTTACTTTC TGGGGTGGAC ATCCAAACCG TTCGATCGAA
4501    AAGTAACGCC ATTTTGCAAG GCATGGAAAA ATACATAACT GAGAATAGAA AAGTTCAGAT CAAGGTCAGG AACAGATGGA ACAGCTGAAT ATGGGCCAAA
        TTCATTGCGG TAAAACGTTC CGTACCTTTT TATGTATTGA CTCTTATCTT TTCAAGTCTA GTTCCAGTCC TTGTCTACCT TGTCGACTTA TACCCGGTTT
4601    CAGGATATCT GTGGTAAGCA GTTCCTGCCC CGGCTCAGGG CCAAGAACAG ATGGAACAGC TGAATATGGG CCAAACAGGA TATCTGTGGT AAGCAGTTCC
        GTCCTATAGA CACCATTCGT CAAGGACGGG GCCGAGTCCC GGTTCTTGTC TACCTTGTCG ACTTATACCC GGTTTGTCCT ATAGACACCA TTCGTCAAGG
4701    TGCCCCGGCT CAGGGCCAAG AACAGATGGT CCCCAGATGC GGTCCAGCCC TCAGCAGTTT CTAGAGAACC ATCAGATGTT TCCAGGGTGC CCCAAGGACC
        ACGGGGCCGA GTCCCGGTTC TTGTCTACCA GGGGTCTACG CCAGGTCGGG AGTCGTCAAA GATCTCTTGG TAGTCTACAA AGGTCCCACG GGGTTCCTGG
4801    TGAAATGACC CTGTGCCTTA TTTGAACTAA CCAATCAGTT CGCTTCTCGC TTCTGTTCGC GCGCTTATGC TCCCCGAGCT CAATAAAAGA GCCCACAACC
        ACTTTACTGG GACACGGAAT AAACTTGATT GGTTAGTCAA GCGAAGAGCG AAGACAAGCG CGCGAATACG AGGGGCTCGA GTTATTTTCT CGGGTGTTGG
4901    CCTCACTCGG GGCGCCAGTC CTCCGATTGA CTGAGTCGCC CGGGTACCCG TGTATCCAAT AAACCCTCTT GCAGTTGCAT CCGACTTGTG GTCTCGCTGT
        GGAGTGAGCC CCGCGGTCAG GAGGCTAACT GACTCAGCGG GCCCATGGGC ACATAGGTTA TTTGGGAGAA CGTCAACGTA GGCTGAACAC CAGAGCGACA
5001    TCCTTGGGAG GGTCTCCTCT GAGTGATTGA CTACCCGTCA GCGGGGGTCT TTCATTTGGG GGCTCGTCCG GGATCGGGAG ACCCCTGCCC AGGGACCACC
        AGGAACCCTC CCAGAGGAGA CTCACTAACT GATGGGCAGT CGCCCCCAGA AAGTAAACCC CCGAGCAGGC CCTAGCCCTC TGGGGACGGG TCCCTGGTGG
5101    GACCCACCAC CGGGAGGTAA GCTGGCCAGC AACTTATCTG TGTCTGTCCG ATTGTCTAGT GTCTATGACT GATTTTATGC GCCTGCGTCG GTACTAGTTA
        CTGGGTGGTG GCCCTCCATT CGACCGGTCG TTGAATAGAC ACAGACAGGC TAACAGATCA CAGATACTGA CTAAAATACG CGGACGCAGC CATGATCAAT
5201    GCTAACTAGC TCTGTATCTG GCGGACCCGT GGTGGAACTG ACGAGTTCGG AACACCCGGC CGCAACCCTG GGAGACGTCC CAGGGACTTC GGGGGCCGTT
        CGATTGATCG AGACATAGAC CGCCTGGGCA CCACCTTGAC TGCTCAAGCC TTGTGGGCCG GCGTTGGGAC CCTCTGCAGG GTCCCTGAAG CCCCCGGCAA
5301    TTTGTGGCCC GACCTGAGTC CTAAAATCCC GATCGTTTAG GACTCTTTGG TGCACCCCCC TTAGAGGAGG GATATGTGGT TCTGGTAGGA GACGAGAACC
        AAACACCGGG CTGGACTCAG GATTTTAGGG CTAGCAAATC CTGAGAAACC ACGTGGGGGG AATCTCCTCC CTATACACCA AGACCATCCT CTGCTCTTGG
5401    TAAAACAGTT CCCGCCTCCG TCTGAATTTT TGCTTTCGGT TTGGGACCGA AGCCGCGCCG CGCGTCTTGT CTGCTGCAGC ATCGTTCTGT GTTGTCTCTG
        ATTTTGTCAA GGGCGGAGGC AGACTTAAAA ACGAAAGCCA AACCCTGGCT TCGGCGCGGC GCGCAGAACA GACGACGTCG TAGCAAGACA CAACAGAGAC
5501    TCTGACTGTG TTTCTGTATT TGTCTGAAAA TATGGGCCCG GGCTAGACTG TTACCACTCC CTTAAGTTTG ACCTTAGGTC ACTGGAAAGA TGTCGAGCGG
        AGACTGACAC AAAGACATAA ACAGACTTTT ATACCCGGGC CCGATCTGAC AATGGTGAGG GAATTCAAAC TGGAATCCAG TGACCTTTCT ACAGCTCGCC
5601    ATCGCTCACA ACCAGTCGGT AGATGTCAAG AAGAGACGTT GGGTTACCTT CTGCTCTGCA GAATGGCCAA CCTTTAACGT CGGATGGCCG CGAGACGGCA
        TAGCGAGTGT TGGTCAGCCA TCTACAGTTC TTCTCTGCAA CCCAATGGAA GACGAGACGT CTTACCGGTT GGAAATTGCA GCCTACCGGC GCTCTGCCGT
5701    CCTTTAACCG AGACCTCATC ACCCAGGTTA AGATCAAGGT CTTTTCACCT GGCCCGCATG GACACCCAGA CCAGGTCCCC TACATCGTGA CCTGGGAAGC
        GGAAATTGGC TCTGGAGTAG TGGGTCCAAT TCTAGTTCCA GAAAAGTGGA CCGGGCGTAC CTGTGGGTCT GGTCCAGGGG ATGTAGCACT GGACCCTTCG
5801    CTTGGCTTTT GACCCCCCTC CCTGGGTCAA GCCCTTTGTA CACCCTAAGC CTCCGCCTCC TCTTCCTCCA TCCGCCCCGT CTCTCCCCCT TGAACCTCCT
        GAACCGAAAA CTGGGGGGAG GGACCCAGTT CGGGAAACAT GTGGGATTCG GAGGCGGAGG AGAAGGAGGT AGGCGGGGCA GAGAGGGGGA ACTTGGAGGA
5901    CGTTCGACCC CGCCTCGATC CTCCCTTTAT CCAGCCCTCA CTCCTTCTCT AGGCGCCCCC ATATGGCCAT ATGAGATCTT ATATGGGGCA CCCCCGCCCC
        GCAAGCTGGG GCGGAGCTAG GAGGGAAATA GGTCGGGAGT GAGGAAGAGA TCCGCGGGGG TATACCGGTA TACTCTAGAA TATACCCCGT GGGGGCGGGG
```

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6001   TTGTAAACTT CCCTGACCCT GACATGACAA GAGTTACTAA CAGCCCCTCT CTCCAAGCTC ACTTACAGGC TCTCTACTTA GTCCAGCACG AAGTCTGGAG
       AACATTTGAA GGGACTGGGA CTGTACTGTT CTCAATGATT GTCGGGGAGA GAGGTTCGAG TGAATGTCCG AGAGATGAAT CAGGTCGTGC TTCAGACCTC
6101   ACCTCTGGCG GCAGCCTACC AAGAACAACT GGACCGACCG GTGGTACCTC ACCCTTACCG AGTCGGCGAC ACAGTGTGGG TCCGCCGACA CCAGACTAAG
       TGGAGACCGC CGTCGGATGG TTCTTGTTGA CCTGGCTGGC CACCATGGAG TGGGAATGGC TCAGCCGCTG TGTCACACCC AGGCGGCTGT GGTCTGATTC
                                                                                                             PmlI
                                                                                                             ----
6201   AACCTAGAAC CTCGCTGGAA AGGACCTTAC ACAGTCCTGC TGACCACCCC CACCGCCCTC AAAGTAGACG GCATCGCAGC TTGGATACAC GCCGCCCACG
       TTGGATCTTG GAGCGACCTT TCCTGGAATG TGTCAGGACG ACTGGTGGGG GTGGCGGGAG TTTCATCTGC CGTAGCGTCG AACCTATGTG CGGCGGGTGC
                                                                                    CD8-Leader
                                                        -----------------------------------------------------------
       PmlI                                             NcoI
       --                                               ------
6301   TGAAGGCTGC CGACCCCGGG GGTGGACCAT CCTCTAGACT GCCATGGCTC TCCCAGTGAC TGCCCTACTG CTTCCCCTAG CGCTTCTCCT GCATGCAGAG
       ACTTCCGACG GCTGGGGCCC CCACCTGGTA GGAGATCTGA CGGTACCGAG AGGGTCACTG ACGGGATGAC GAAGGGGATC GCGAAGAGGA CGTACGTCTC
                                                                   VH
       ---------------------------------------------------------------------------------------------------------
6401   GTGAAGCTGC AGGAGTCAGG GGGAGGCTTC GTGAAGCCTG GAGGGTCCCT CAAAGTCTCC TGTGCAGCCT CTGGATTCAC TTTCAGTAGC TATGCCATGT
       CACTTCGACG TCCTCAGTCC CCCTCCGAAG CACTTCGGAC CTCCCAGGGA GTTTCAGAGG ACACGTCGGA GACCTAAGTG AAAGTCATCG ATACGGTACA
                                                                   VH
       ---------------------------------------------------------------------------------------------------------
6501   CCTGGGTTCG CCTGAGTCCG GAGATGAGGC TGGAGTGGGT CGCAACCATT AGCAGTGCTG GTGGTTACAT CTTCTATTCT GACAGTGTGC AGGGACGATT
       GGACCCAAGC GGACTCAGGC CTCTACTCCG ACCTCACCCA GCGTTGGTAA TCGTCACGAC CACCAATGTA GAAGATAAGA CTGTCACACG TCCCTGCTAA
                                                                   VH
       ---------------------------------------------------------------------------------------------------------
6601   CACCATTTCC AGAGACAATG CCAAGAACAC CCTGCACCTG CAAATGGGCA GTCTGAGGTC TGGGGACACG GCCATGTATT ACTGTGCAAG GCAGGGATTT
       GTGGTAAAGG TCTCTGTTAC GGTTCTTGTG GGACGTGGAC GTTTACCCGT CAGACTCCAG ACCCCTGTGC CGGTACATAA TGACACGTTC CGTCCCTAAA
                                                                                    (G4S)3 Serine-glycine linker
                                                                                    ------------------------------
                                    VH
       ---------------------------------------------------------------------
6701   GGTAACTACG GTGATTACTA TGCTATGGAC TACTGGGGCC AAGGGACCAC GGTCACCGTC TCCTCAGGTG GAGGTGGATC AGGTGGAGGT GGATCTGGTG
       CCATTGATGC CACTAATGAT ACGATACCTG ATGACCCCGG TTCCCTGGTG CCAGTGGCAG AGGAGTCCAC CTCCACCTAG TCCACCTCCA CCTAGACCAC
                                                                   VL
                   -----------------------------------------------------------------------------------------
(G4S)3 Serine-glycine linker
       ------------
6801   GAGGTGGATC TGACATTGAG CTCACCCAGT CTCCATCCTC CCTGGCTGTG TCAGCAGGAG AGAAGGTCAC TATGAGCTGC AAATCCAGTC AGAGTCTGCT
       CTCCACCTAG ACTGTAACTC GAGTGGGTCA GAGGTAGGAG GGACCGACAC AGTCGTCCTC TCTTCCAGTG ATACTCGACG TTTAGGTCAG TCTCAGACGA
                                                                   VL
       ---------------------------------------------------------------------------------------------------------
6901   CAACAGTAGA ACCCGAAAGA ACCAGTTGGC TTGGTACCAG CAAAAACCAG GACAGTCTCC TGAACTGCTG ATCTACTGGG CATCCACTAG GCAATCTGGA
       GTTGTCATCT TGGGCTTTCT TGGTCAACCG AACCATGGTC GTTTTTGGTC CTGTCAGAGG ACTTGACGAC TAGATGACCC GTAGGTGATC CGTTAGACCT
                                                                   VL
```

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        ----------------------------------------------------------------------------------------------------
7001    GTCCCTGATC GCTTCACAGG CAGTGGATCT GGGACAGATT TCACTCTCAC CATCAGCAGT GTGCAGGCTG AAGACCTGGC AGTTTATTAC TGCCAGCAAT
        CAGGGACTAG CGAAGTGTCC GTCACCTAGA CCCTGTCTAA AGTGAGAGTG GTAGTCGTCA CACGTCCGAC TTCTGGACCG TCAAATAATG ACGGTCGTTA
                            VL                                                              CD8 transmembrane domain
        -------------------------------------------------          ----------------------------------------
                                                               NotI
                                                             --------
7101    CTTATAATCT ACTCACGTTC GGTCCTGGGA CCAAGCTGGA GATCAAACGG GCGGCCGCAC CCACCACGAC GCCAGCGCCG CGACCACCAA CCCCGGCGCC
        GAATATTAGA TGAGTGCAAG CCAGGACCCT GGTTCGACCT CTAGTTTGCC CGCCGGCGTG GGTGGTGCTG CGGTCGCGGC GCTGGTGGTT GGGGCCGCGG
                                                Cd8 transmembrane domain
        ----------------------------------------------------------------------------------------------------
7201    CACGATCGCG TCGCAGCCCC TGTCCCTGCG CCCAGAGGCG TGCCGGCCAG CGGCGGGGGG CGCAGTGCAC ACGAGGGGGC TGGACTTCGC CTGTGATATC
        GTGCTAGCGC AGCGTCGGGG ACAGGGACGC GGGTCTCCGC ACGGCCGGTC GCCGCCCCCC GCGTCACGTG TGCTCCCCCG ACCTGAAGCG GACACTATAG
                                Cd8 transmembrane domain
        --------------------------------------------------------------------------
                                                                                  CD3 zeta chain intracellular domain
                                                                                  --------------------------
7301    TACATCTGGG CGCCCTTGGC CGGGACTTGT GGGGTCCTTC TCCTGTCACT GGTTATCACC CTTTACTGCA ACCACAGAGT GAAGTTCAGC AGGAGCGCAG
        ATGTAGACCC GCGGGAACCG GCCCTGAACA CCCCAGGAAG AGGACAGTGA CCAATAGTGG GAAATGACGT TGGTGTCTCA CTTCAAGTCG TCCTCGCGTC
                                             CD3 zeta chain intracellular domain
        ----------------------------------------------------------------------------------------------------
7401    AGCCCCCCGC GTACCAGCAG GGCCAGAACC AGCTCTATAA CGAGCTCAAT CTAGGACGAA GAGAGGAGTA CGATGTTTTG GACAAGAGAC GTGGCCGGGA
        TCGGGGGGCG CATGGTCGTC CCGGTCTTGG TCGAGATATT GCTCGAGTTA GATCCTGCTT CTCTCCTCAT GCTACAAAAC CTGTTCTCTG CACCGGCCCT
                                             CD3 zeta chain intracellular domain
        ----------------------------------------------------------------------------------------------------
7501    CCCTGAGATG GGGGGAAAGC CGAGAAGGAA GAACCCTCAG GAAGGCCTGT ACAATGAACT GCAGAAAGAT AAGATGGCGG AGGCCTACAG TGAGATTGGG
        GGGACTCTAC CCCCCTTTCG GCTCTTCCTT CTTGGGAGTC CTTCCGGACA TGTTACTTGA CGTCTTTCTA TTCTACCGCC TCCGGATGTC ACTCTAACCC
                                             CD3 zeta chain intracellular domain
        ----------------------------------------------------------------------------------------------------
7601    ATGAAAGGCG AGCGCCGGAG GGGCAAGGGG CACGATGGCC TTTACCAGGG TCTCAGTACA GCCACCAAGG ACACCTACGA CGCCCTTCAC ATGCAGGCCC
        TACTTTCCGC TCGCGGCCTC CCCGTTCCCC GTGCTACCGG AAATGGTCCC AGAGTCATGT CGGTGGTTCC TGTGGATGCT GCGGGAAGTG TACGTCCGGG
        CD3 zeta chain intracellular domain
        ----------          XhoI
                            ------
7701    TGCCCCCTCG CTAACAGCCA CTCGAG
        ACGGGGGAGC GATTGTCGGT GAGCTC
```

Figure 18 top strand: SEQ ID NO:37
Figure 19 bottom strand: SEQ ID NO:38

Figure 18 (5 of 5)

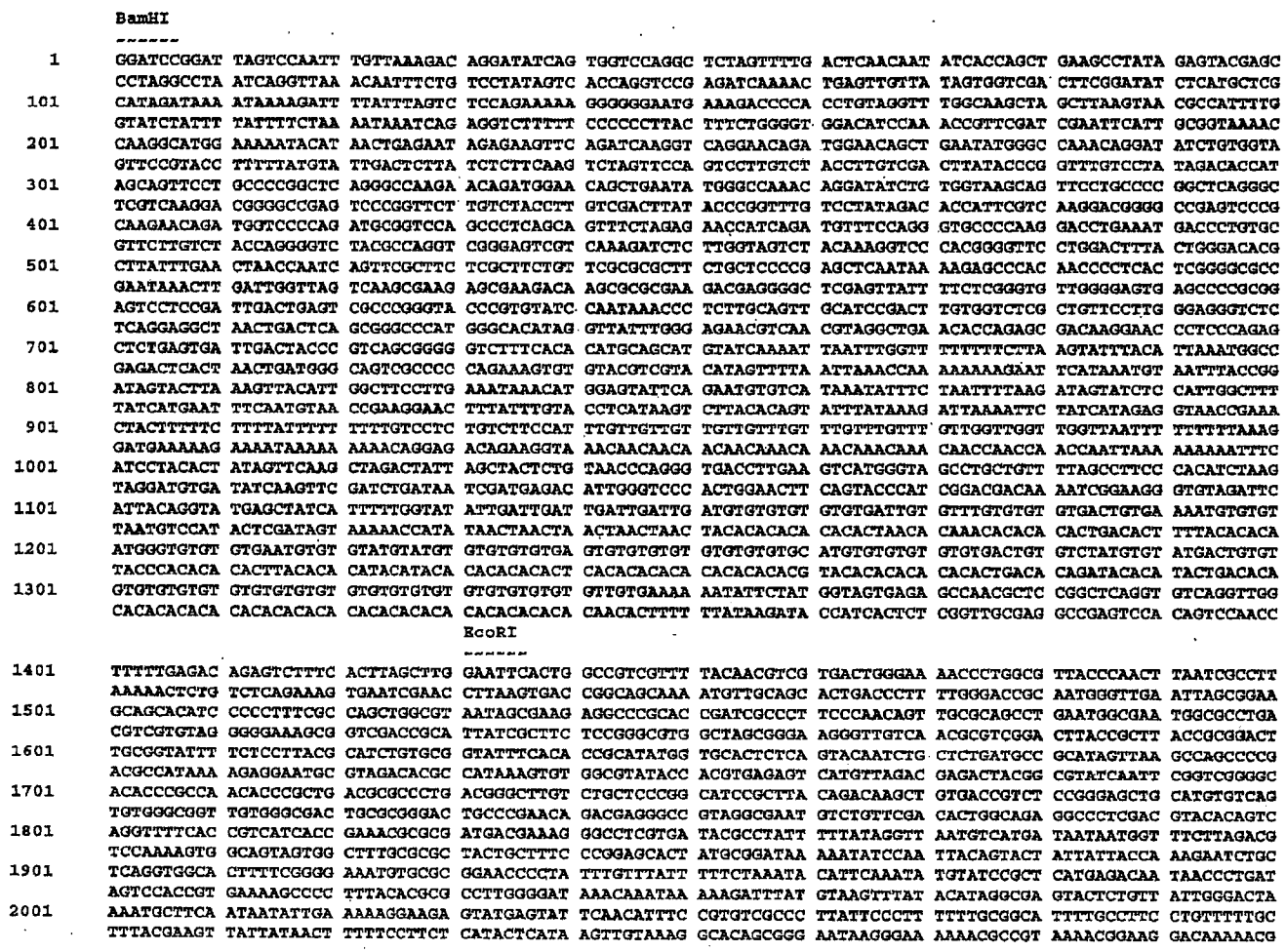

Figure 19 (1 of 6)

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      AGTGGGTCTT TGCGACCACT TTCATTTTCT ACGACTTCTA GTGCTCACCC AATGTAGCTT GACCTAGAGT TGTCGCCATT CTAGGAACTC
2201  AGTTTTCGCC CCGAAGAACG TTTTCCAATG ATGAGCACTT TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTATTGA CGCCGGGCAA GAGCAACTCG
      TCAAAAGCGG GGCTTCTTGC AAAAGTTTAC TACTCGTGAA AATTTCAAGA CGATACACCG CGCCATAATA GGGCATAACT GCGGCCCGTT CTCGTTGAGC
2301  GTCGCCGCAT ACACTATTCT CAGAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC ATCTTACGGA TGGCATGACA GTAAGAGAAT TATGCAGTGC
      CAGCGGCGTA TGTGATAAGA GTCTTACTGA ACCAACTCAT GAGTGGTCAG TGTCTTTTCG TAGAATGCCT ACCGTACTGT CATTCTCTTA ATACGTCACG
2401  TGCCATAACC ATGAGTGATA ACACTGCGGC CAACTTACTT CTGACAACGA TCGGAGGACC GAAGGAGCTA ACCGCTTTTT TGCACAACAT GGGGGATCAT
      ACGGTATTGG TACTCACTAT TGTGACGCCG GTTGAATGAA GACTGTTGCT AGCCTCCTGG CTTCCTCGAT TGGCGAAAAA ACGTGTTGTA CCCCCTAGTA
2501  GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAAA CGACGAGCGT GCTGCTCGCA CTGTTGGTGCT ACGGACATCG TTACCGTTGT TGCAAGCGCT ATTGACAGTC
      CATTGAGCGG AACTAGCAAC CCTTGGCCTC GACTTACTTC GGTATGGTTT GCTGCTCGCA CTGTTGGTGCT ACGGACATCG TTACCGTTGT TGCAAGCGCT ATTGACAGTC
2601  AACTATTAAC TGGCGAACTA CTTACTCTAG CTTCCCGGCA ACAATTAATA GACTGGATGG AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT
      TTGATAATTG ACCGCTTGAT GAATGAGATC GAAGGGCCGT TGTTAATTAT CTGACCTACC TCCGCCTAAT TCAACGTCCT GGTGAAGACG CGAGCCGGGA
2701  TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT CATTGCAGCA CTGGGGCCAG ATGGTAAGCC CTCCCGTATC
      AGGCCGACCG ACCAAATAAC GACTATTTAG ACCTCGGCCA CTCGCACCCA GAGCGCCATA GTAACGTCGT GACCCCGGTC TACCATTCGG GAGGGCATAG
2801  GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACTGTCAG
      CATCAATAGA TGTGCTGCCC CTCAGTCCGT TGATACCTAC TTGCTTTATC TGTCTAGCGA CTCTATCCAC GGAGTGACTA ATTCGTAACC ATTGACAGTC
2901  ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA TGACCAAAAT
      TGGTTCTAAAT GAGTATATAT GAATCTAACT TAAATTTTGA AGTAAAAAAT AAATTTCCCT AGATCCACTT CTAGGAAAAA CTATTAGAGT ACTGGTTTTA
3001  CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG
      GGGAATTGCA CTCAAAAGCA AGGTGACTCG CAGTCTGGGG CATCTTTTCT AGTTCCTAG AAGAACTCTA GGAAAAAAAG ACGCGCATTA GACGACGAAC
3101  CAAACAAAAA AACCACCGCT ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG CTTCAGCAGA GCGCAGATAC
      GTTTGTTTTT TTGGTGGCGA TGGTCGCCAC CAAACAAACG GCCTAGTTCT CGATGGTTGA GAAAAAGGCT TCCATTGACC GAAGTCGTCT CGCGTCTATG
3201  CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC
      GTTTATGACA GGAAGATCAC ATCGGCATCA ATCCGGTGGT GAAGTTCTTG AGACATCGTG GCGGATGTAT GGAGCGAGAC GATTAGGACA ATGGTCACCG
3301  TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA
      ACGACGGTCA CCGCTATTCA GCCACAACTG AGTTCTGCTA TCAATGGCCT ATTCCGCGTC GCCAGCCCGA CTTGCCCCCC AAGCACGTGT
3401  CAGCCCAGCT TGGAGCGAAC GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCATTGA GAAAGCGCCA CGCTTCCCGA AGGGAGAAAG GCGGACAGGT
      GTCGGGTCGA ACCTCGCTTG CTGGATGTGG CTTGACTCTA TGGATGTCGC ACTCGTAACT CTTTCGCGGT GCGAAGGGCT TCCCTCTTTC CGCCTGTCCA
3501  ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG
      TAGGCCATTC GCCGTCCCAG CCTTGTCCTC TCGCGTGCTC CCTCGAAGGT CCCCCTTTGC GGACCATAGA AATATCAGGA CAGCCCAAAG CGGTGGAGAC
3601  ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT
      TGAACTCGCA GCTAAAAACA CTACGAGCAG TCCCCCGCCC TCGGATACCT TTTTGCGGTC GTTGCGCCGG AAAAATGCCA AGGACCGGAA AACGACCGAA
3701  TTTGCTCACA TGTTCTTCCT GCGTTATCCC CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG CTGATACCGC TCGCCGCAGC CGAACGACCG
      AAACGAGTGT ACAAGAAAGG GGACTAAGAC ACCTATTGGC ATAATGGCGG AAACTCACTC AGCGGCGTCG AGCGGCGTCG GCTTGCTGGC
3801  AGCGCAGCGA GTCAGTGAGC GAGGAAGCGG AAGAGCGCCC AATACGCAAA CCGCCTCTCC CCGCGCGTTG GCCGATTCAT TAATGCAGCT GGCACGACAG
      TCGCGTCGCT CAGTCACTCG CTCCTTCGCC TTCTCGCGGG TTATGCGTTT GGCGGAGAGG GGCGCGCAAC CGGCTAAGTA ATTACGTCGA CCGTGCTGTC
3901  GTTTCCCGAC TGGAAAGCGG GCAGTGAGCG CAACGCAATT AATGTGAGTT AGCTCACTCA TTAGGCACCC CAGGCTTTAC ACTTTATGCT TCCGGCTCGT
      CAAAGGGCTG ACCTTTCGCC CGTCACTCGC GTTGCGTTAA TTACACTCAA TCGAGTGAGT AATCCGTGGG GTCCGAAATG TGAAATACGA AGGCCGAGCA
4001  ATGTTGTGTG GAATTGTGAG CGGATAACAA TTTCACACAG GAAACAGCTA TGACCATGAT TACGCCAAGC TTTGCTCTTA GGAGTTTCCT AATACATCCC
      TACAACACAC CTTAACACTC GCCTATTGTT AAAGTGTGTC CTTTGTCGAT ACTGGTACTA ATGCGGTTCG AAACGAGAAT CCTCAAAGGA TTATGTAGGG
4101  AAACTCAAAT ATATAAAGCA TTTGACTTGT TCTATGCCCT AGGGGGCGGG GGGAAGCTAA GCCAGCTTTT TTTAACATTT AAAATGTTAA TTCCATTTTA
      TTTGAGTTTA TATATTTCGT AAACTGAACA AGATACGGGA TCCCCCGCCC CCCTTCGATT CGGTCGAAAA AAATTGTAAA TTTTACAATT AAGGTAAAAT
4201  AATGCACAGA TGTTTTTATT TCATAAGGGT TTCAAATGTGC ATGAATGCTG CAATATTCCT GTTACCAAAG CTAGTATAAA TAAAAATAGA TAAACGTGGA
      TTACGTGTCT ACAAAATAA AGTATTCCCA AAGTTACACG TACTTACGAC GTTATAAGGA CAATGGTTTC GATCATATTT ATTTTTATCT ATTTGCACCT
```

Figure 19 (2 of 6)

```
4301  AATTACTTAG AGTTTCTGTC ATTAACGTTT CCTTCCTCAG TTGACAACAT AAATGCGCTG CTGAGCAAGC CAGTTTGCAT CTGTCAGGAT CAATTTCCCA
      TTAATGAATC TCAAAGACAG TAATTGCAAA GGAAGGAGTC AACTGTTGTA TTTACGCGAC GACTCGTTCG GTCAAACGTA GACAGTCCTA GTTAAAGGGT
4401  TTATGCCAGT CATATTAATT ACTAGTCAAT TAGTTGATTT TTATTTTTGA CATATACATG TGAATGAAAG ACCCCACCTG TAGGTTTGGC AAGCTAGCTT
      AATACGGTCA GTATAATTAA TGATCAGTTA ATCAACTAAA AATAAAAACT GTATATGTAC ACTTACTTTC TGGGGTGGAC ATCCAAACCG TTCGATCGAA
4501  AAGTAACGCC ATTTTGCAAG GCATGGAAAA ATACATAACT GAGAATAGAA AAGTTCAGAT CAAGGTCAGG AACAGATGGA ACAGCTGAAT ATGGGCCAAA
      TTCATTGCGG TAAAACGTTC CGTACCTTTT TATGTATTGA CTCTTATCTT TTCAAGTCTA GTTCCAGTCC TTGTCTACCT TGTCGACTTA TACCCGGTTT
4601  CAGGATATCT GTGGTAAGCA GTTCCTGCCC CGGCTCAGGG CCAAGAACAG ATGGAACAGC TGAATATGGG CCAAACAGGA TATCTGTGGT AAGCAGTTCC
      GTCCTATAGA CACCATTCGT CAAGGACGGG GCCGAGTCCC GGTTCTTGTC TACCTTGTCG ACTTATACCC GGTTTGTCCT ATAGACACCA TTCGTCAAGG
4701  TGCCCCCGGCT CAGGGCCAAG AACAGATGGT CCCCAGATGC GTCCCAGCCC TCAGCAGTTT CTAGAGAACC ATCAGATGTT TCCAGGGTGC CCCAAGGACC
      ACGGGGCCGA GTCCCGGTTC TTGTCTACCA GGGGTCTACG CCAGGTCGGG AGTCGTCTAA GATCTCTTGG TAGTCTACAA AGGTCCCACG GGGTTCCTGG
4801  TGAAATGACC CTGTGCCTTA TTTGAACTAA CCAATCAGTT CGCTTCTCGC TTCTGTTCGC GCGCTTATGC TCCCCGAGCT CAATAAAAGA GCCCACAACC
      ACTTTACTGG GACACGGAAT AAACTTGATT GGTTAGTCAA GCGAAGAGCG AAGACAAGCG CGCGAATACG AGGGGCTCGA GTTATTTTCT CGGGTGTTGG
4901  CCTCACTCGG GGCACCAGTC CTCCGATTGA CTGAGTCGCC CGGGTACCCG TGTATCCAAT AAACCCTCTT GCAGTTGCAT CCGACTTGTG GTCTCGCTGT
      GGAGTGAGCC CCGCGAGTCAG GAGGCTAACT GACTCAGCGG GCCCATGGGC ACATAGGTTA TTTGGGAGAA CGTCAACGTA GGCTGAACAC CAGAGCGACA
5001  TCCTTGGGAG GGTCTCCTCT GAGTGATTGA CTACCCGTCA GCGGGGGTCT TTCATTTGGG GGCTCGTCCG GGATCGGGAG ACCCCTGCCC AGGGACCACC
      AGGAACCCTC CCAGAGGAGA CTCACTAACT GATGGGCAGT CGCCCCCAGA AAGTAAACCC CCGAGCAGGC CCTAGCCCTC TGGGGACGGG TCCCTGGTGG
5101  GACCCACCAC CGGGAGGTAA GCTGGCCAGC AACTTATCTG TGTCTGTCCG ATTGTCTAGT GTCTATGACT GATTTTATGC GCCTGCGTCG GTACTAGTTA
      CTGGGTGGTG GCCCTCCATT CGACCGGTCG TTGAATAGAC ACAGACAGGC TAACAGATCA CAGATACTGA CTAAAATACG CGGACGCAGC CATGATCAAT
5201  GCTAACTAGC TCTGTATCTG GCGGACCCGT GGTGGAACTG ACGAGTTCGG AACACCCGGC CGCAACCCTG GCGAGACGTCC CAGGGACTTC GGGGGCCGTT
      CGATTGATCG AGACATGAAC CGCCTGGGCA CCACCTTGAC TGCTCAAGCC TTGTGGGCCG GCGTTGGGAC CCTCTGCAGG GTCCCTGAAG CCCCCGGCAA
5301  TTTGTGGCCC GACCTGAGTC CTAAAATCCC GATCGTTTAG GACTCTTTGG TGCACCCCCC TTAGAGGAGG GATATGTGGT TCTGGTAGGA GACGAGAACC
      AAACACCGGG CTGGACTCAG GATTTAGGGG CTAGCAAATC CTGAGAAACC ACGTGGGGGG AATCTCCTCC CTATACACCA AGACCATCCT CTGCTCTTGG
5401  TAAAACAGTT CCCGCCTCCG TCTGAATTTT TGCTTTCGGT TTGGGACCGA AGCCGCGCCG CGCGTCTTGT CTGCTGCAGC ATCGTTCTGT GTTGTCTCTG
      ATTTTGTCAA GGGCGGAGGC AGACTTAAAA ACGAAAGCCA AACCCTGGCT TCGGCGCGGC GCGCAGAACA GACGACGTCG TAGCAAGACA CAACAGACGC
5501  TCTGACTGTG TTTCTGTATT TGTCTGGAAA TATGGGCCCG CCGATCTGAC AATGGTGAGG GAATTCAAAC TTAAGTTTG ACCTTAGGTC ACTGGAAAGA TGTCGAGCGG
      AGACTGACAC AAAGACATAA ACAGACTTTT ATACCCGGGC CCGATCTGAC AATGGTGAGG GAATTCAAAC TGGAATCCAG TGACCTTTCT ACAGCTCGCC
5601  ATCGCTCACA ACCAGTCGGT AGATGTCAAG AAGAGACGTT GGGTTACCTT CTGCTCTGCA GAATGGCCAA CCTTTAACGT CGGATGGCCG CGAGACGGCA
      TAGCGAGTGT TGGTCAGCCA TCTACAGTTC TTCTCTGCAA CCCAATGGAA GACGAGACGT CTTACCGGTT GGAAATTGCA GCCTACCGGC GCTCTGCCGT
5701  CCTTTAACCG AGACCTCATC ACCCAGGTTA AGATCAAGGT CTTTTCACCT GGCCCGCATG GACACCCAGA CCAGGTCCCC TACATCGTGA CCTGGGAAGC
      GGAAATTGGC TCTGGAGTAG TGGGTCCAAT TCTAGTTCCA GAAAAGTGGA CCGGGCGTAC CTGTGGGTCT GGTCCAGGGG ATGTAGCACT GGACCCTTCG
5801  CTTGGCTTTT GACCCCCCTC CCTGGGTCAA GCCCTTTGTA CACCCTAAGC CTCCGCCTCC TCTTCCTCCA TCCGCCCGT CTCTCCCCCT TGAACCTCCT
      GAACCGAAAA CTGGGGGGAG GGACCCAGTT CGGGAAACAT GTGGGATTCG GAGGCGGAGG AGAAGGAGGT AGGCGGGGCA GAGAGGGGGA ACTTGGAGGA
5901  CGTTCGACCC CGCCTCGATC CTCCCTTTAT CCAGCCCTCA CTCCTTCTCT AGGCGCCCCC ATATGGCCAT ATGAGATCTT ATATGGGGCA CCCCGCCCCC
      GCAAGCTGGG GCGAAGCTAG GAGGGAAATA GGTCGGGAGT GAGGAAGAGA TCCGCGGGGG TATACCGGTA TACTCTAGAA TATACCCCGT GGGGGCGGGG
6001  TTGTAAACTT CCCTGACCCT CATGTTACTAA GAGTTACTAA CAGCCCCTCT CTCCAAGCTC ACTTACAGGC TCTCTACTTA GTCCAGCACG AAGTCTGGAG
      AACATTTGAA GGGACTGGGA CTGTACTGTT CTCAATGATT GTCGGGGAGA GAGGTTCGAG TGAATGTCCG AGAGATGAAT CAGGTCGTGC TTCAGACCTC
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Figure 19 (3 of 6)

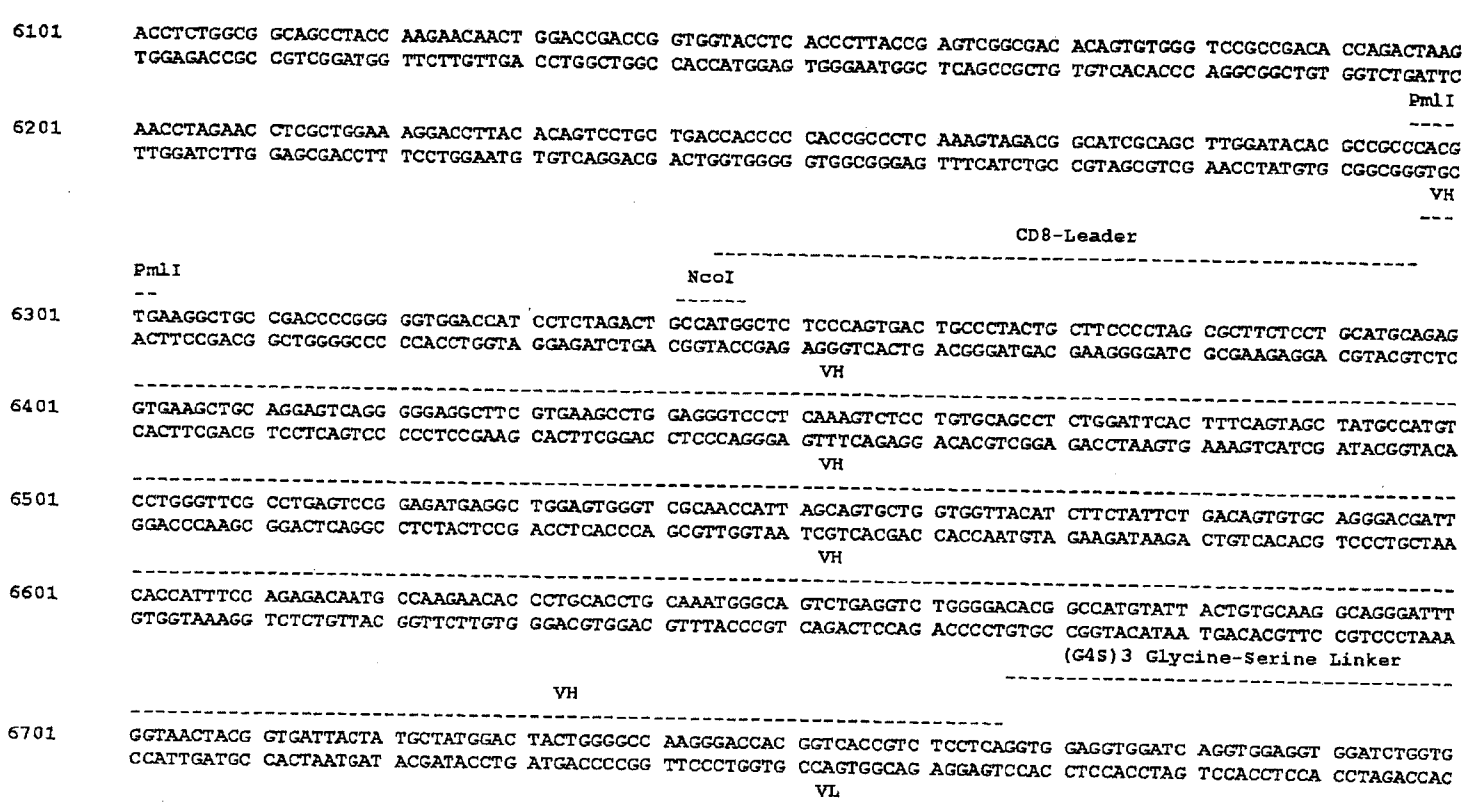


Figure 19 (4 of 6)

Figure 19 (5 of 6)

```
                                                                                                                    
7701    TTGGGATGAA AGGCGAGCGC CGGAGGGGCA AGGGGCACGA TGGCCTTTAC CAGGGTCTCA GTACAGCCAC CAAGGACACC TACGACGCCC TTCACATGCA
        AACCCTACTT TCCGCTCGCG GCCTCCCCGT TCCCCGTGCT ACCGGAAATG GTCCCAGAGT CATGTCGGTG GTTCCTGTGG ATGCTGCGGG AAGTGTACGT
        CD3 zeta chain intracellular domain
        -----------------
                                    XhoI
                                   -------
7801    GGCCCTGCCC CCTCGCTAAC AGCCACTCGA G
        CCGGGACGGG GGAGCGATTG TCGGTGAGCT C
```

Figure 19 top strand: SEQ ID NO:39
Figure 19 bottom strand: SEQ ID NO:40

Figure 19 (6 of 6)

Figure 20A

1. Mouse MUC16-CD Peptide 1 (SEQ ID NO:21):

TLDRKSVFVDGYSQNRDD **19 AA**

2. Mouse 1st Cysteine Loop peptide 2 (SEQ ID NO:22):

KSYFSDCQVLAFRSVSNNNNHTGVDSLCNFSPL **33 AA**

3. Mouse 2nd Cysteine Loop peptide 3 (SEQ ID NO:23):

SLYSNCRLASLRPKKNGTATGVNAICSYHQN **32 AA**

Figure 20B

Alignment of mouse MUC16 (SEQ ID NO:24) and human MUC16 (SEQ ID NO:25) amino acid sequences

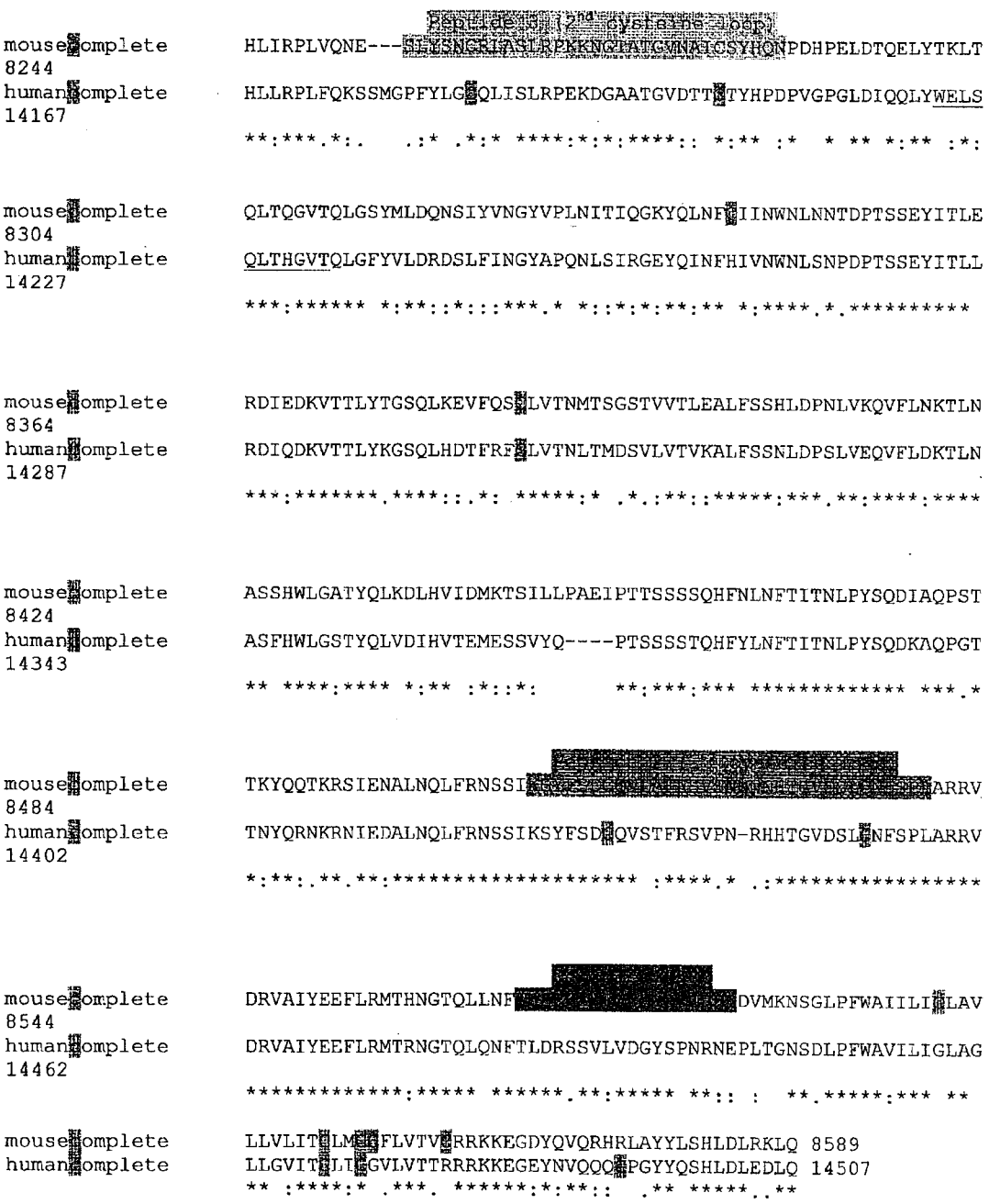
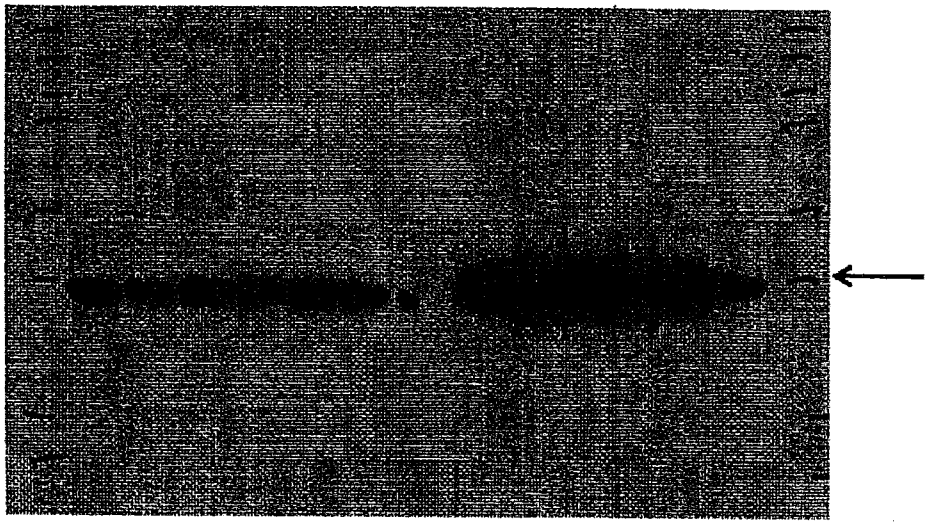


Figure 21

Mouse MUC16 CD Peptide 1

IDI 9F7 16A9 21A7 24G10 10C4 17F2 1A8 1F8 12B10 17H10 18D5 23B12
1 2 3 4 5 6 7 8 9 10 11 12 13



Mouse MUC16 CL Peptide 3

25E9 16F12 4A6 5D1 21B8 21E1 8A2 13E5 23G4 21D3 FB XX 4H11hu
14 15 16 17 18 19 20 21 22 23 24 25 26

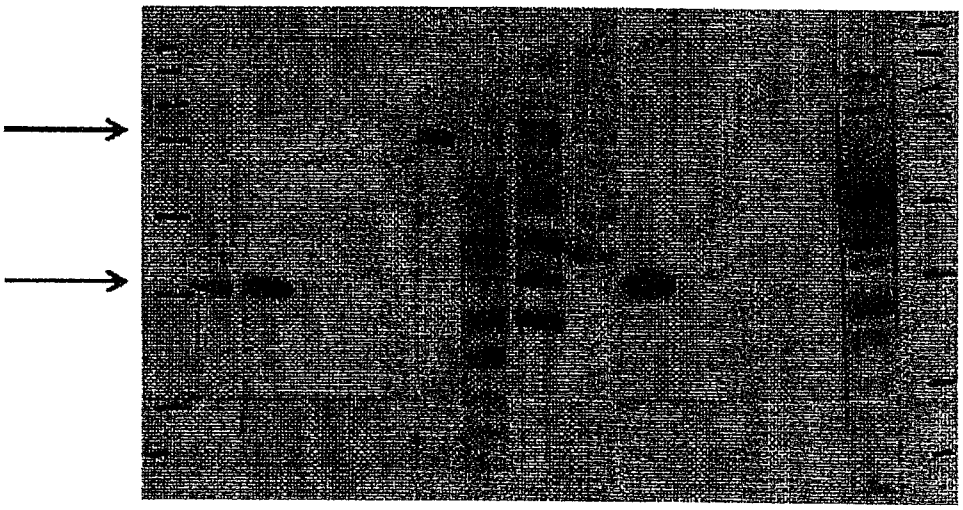


Figure 22

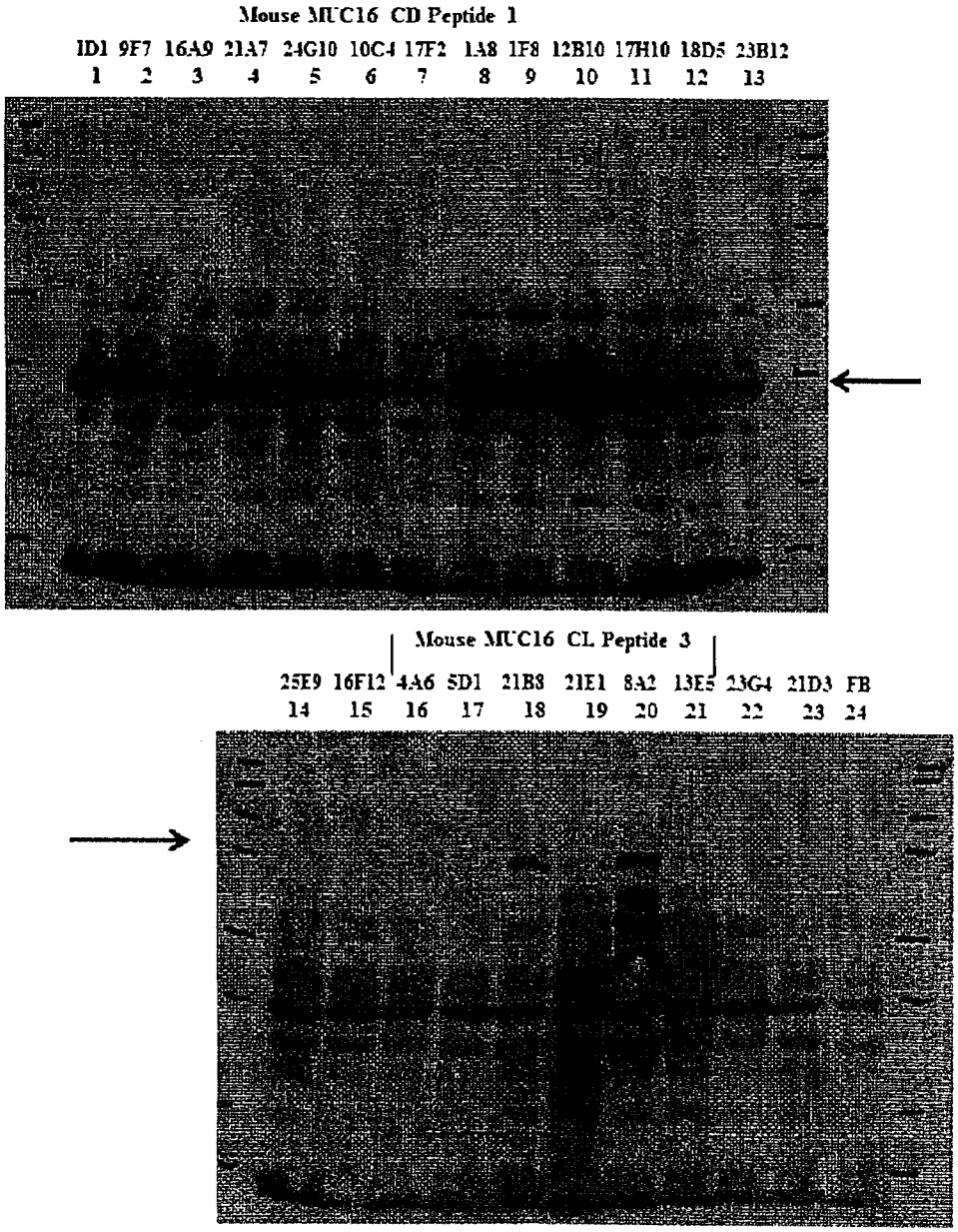
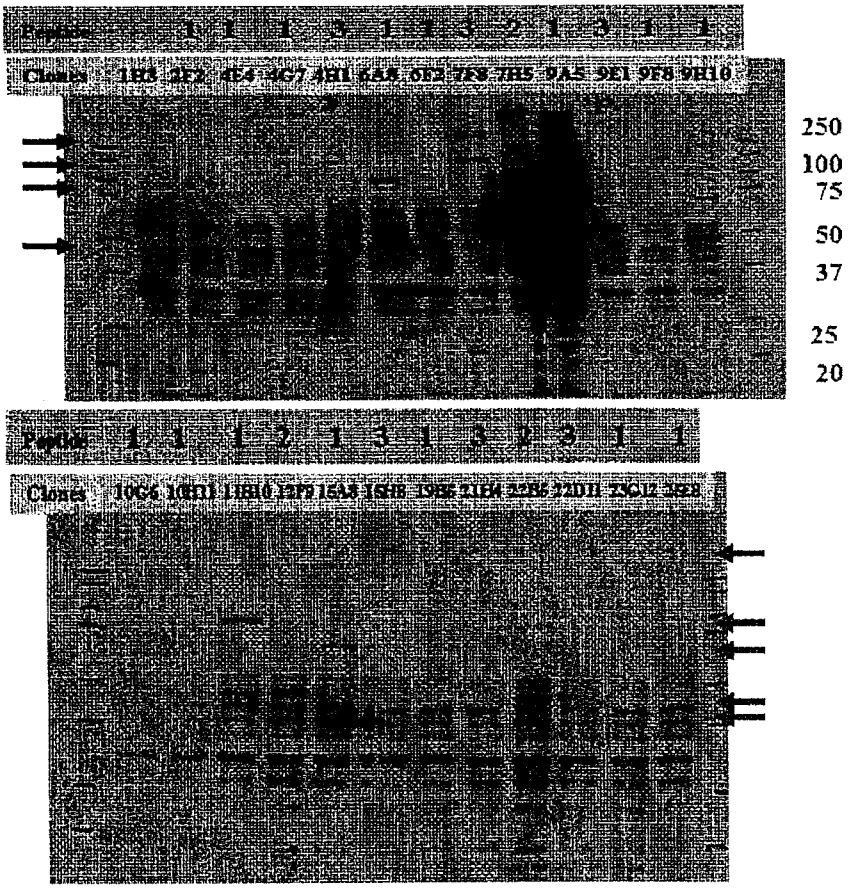

Mouse MUC16 CD Peptide 1
ID1 9F7 16A9 21A7 24G10 10C4 17F2 1A8 1F8 12B10 17H10 18D5 23B12
1 2 3 4 5 6 7 8 9 10 11 12 13
Mouse MUC16 CL Peptide 3
25E9 16F12 4A6 5D1 21B8 21E1 8A2 13E5 23G4 21D3 FB
14 15 16 17 18 19 20 21 22 23 24

Figure 23


Peptide 1 1 1 2 1 3 1 3 2 3 1 1
Clones 1H3 2E2 4C4 4C7 4H1 6A8 6E2 7F8 7H5 9A5 9E1 9F8 9H10
250
100
75
50
37
25
20
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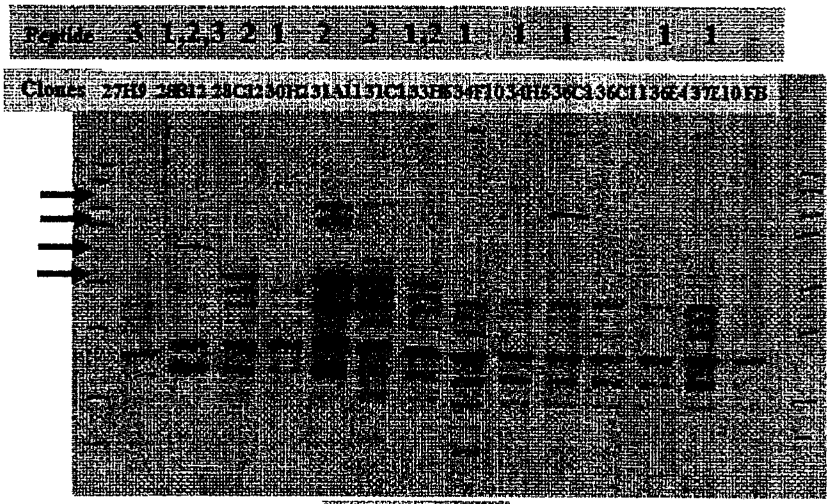
Clones 10C4 10E11 11H10 12F9 16A8 16H8 19D6 21B4 22B6 22D11 23C12 24E8


Peptide 3 1,2,3 2 1 2 2 1,2 1 1 1 - 1 1 -
Clones 27H9 28B12 28C12 30H2 31A11 31C1 33H6 34F10 34H5 36C1 36C11 36E4 37E10 FB

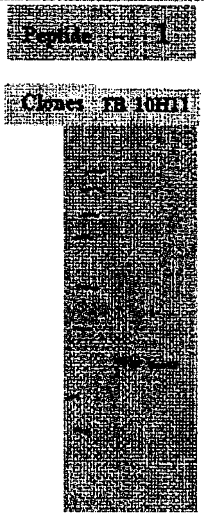

Peptide - 1
Clones FB 10H11

Figure 24

A. Nucleotide sequence encoding 12B10.3G10-V_H (SEQ ID NO:26)

GAGGTGAAGCTGGAGGAGTCAGGTGGAGGATTGGTGCAGCCTAAAGGATCATTGAAACTCTCATGTGCCGCCTCT
GGTTTCACCTTCAATACCTATGCCGTGCACTGGGTCCGCCAGGCTCCAGGAAAGGGTATGGAATGGGTTGCTCGC
ATAAGAAGTAAAAGTGGAAATTATGCAACATATTATGCGATTCAGTGAAAGACAGATTCACCATCTCCAGAAAT
GATTCACAGAGCATGCTCTATCTGCAAATGAACAACCTGAAAACTGAGGACACAGCCATATATTACTGTGTGAGA
GCGGGTAACAACGGGGCCTTTCCTTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA

B. 12B10.3G10-V_H Amino Acid sequence (SEQ ID NO:27)

EVKLEESGGGLVQPKGSLKLSCAASGFTFNTYAVHWVRQAPGKGMEWVARIRSKSGNYAT
YYADSVKDRFTISRNDSQSMLYLQMNNLKTEDTAIYYCVRAGNNGAFPYWGQGTTVTVSS

C. Nucleotide sequence encoding 12B10.3G10-V_L (SEQ ID NO:28)

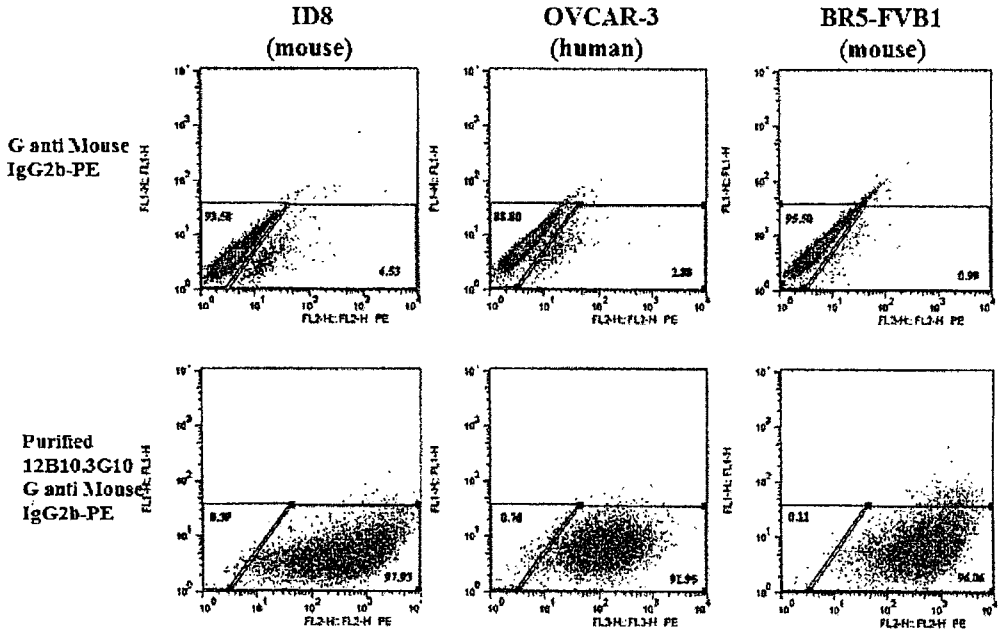
Note the VL has an optional *NotI* site added by the primer for cloning.

GACATTGAGCTCACCCAGTCTCCATCCTCACTGTCTGCATCTCTGGGAGGCAGAGTCACCATCACTTGCAAGGCT
AGCCAAGATATTAAGAAGTATATAGCTTGGTACCAACACAAGCCTGGAAAAACTCCTCGACTACTCATACATTTC
ACATCTACATTACAGACAGGCATCCCATCAAGGTTCAGTGGACGTGGGTCTGGGAGAGACTATTCCTTCAGCATC
AGCAACCTGGAGTCTGAAGATATTGCAACTTATTATTGTCTACAGTATGATAGTCTGTACACGTTCGGAGGGGGG
ACCAAGCTGGAGATCAAACGGGCGGCCGCA

D. 12B10.3G10-V_L Amino Acid sequence (SEQ ID NO:29)

DIELTQSPSSLSASLGGRVTITCKASQDIKKYIAWYQHKPGKTPRLLIHFTSTLQTGIPS
RFSGRGSGRDYSFSISNLESEDIATYYCLQYDSLYTFGGGTKLEIKRAAA

Figure 25


ID8
(mouse)
OVCAR-3
(human)
BR5-FVB1
(mouse)
G anti Mouse
IgG2b-PE
Purified
12B10.3G10
G anti Mouse
IgG2b-PE

Peptide 1 near Cleavage Site:
NFSPLARRVDRVAIYEE (SEQ ID NO:01)

Peptide 2 before Transmembrane:
TLDRSSVLVDGYSPNRNE (SEQ ID NO:02)

Peptide 3 inside Transmembrane:
CGVLVTTRRRKKEGEYNVQQQ (SEQ ID NO:03)