Yeast-MUC1 Immunotherapeutic Compositions and Uses Thereof

GOVERNMENT RIGHTS

[0001] This invention was created in the performance of a Cooperative Research and Development Agreement with the National Institutes of Health, an Agency of the Department of Health and Human Services. The Government of the United States has certain rights in this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 61/524,407, filed August 17, 2011. The entire disclosure of U.S. Provisional Patent Application No. 61/524,407 is incorporated herein by reference.

STATEMENT REGARDING JOINT RESEARCH AGREEMENT

[0003] This invention was made by or on behalf of parties to a Cooperative Research and Development Agreement, executed May 8, 2008. The parties to the Cooperative Research and Development Agreement are: GlobeImmune, Inc. and the U.S. Department of Health and Human Services, as represented by National Cancer Institute, an Institute, Center or Division of the National Institutes of Health.

REFERENCE TO A SEQUENCE LISTING

[0004] This application contains a Sequence Listing submitted electronically as a text file by EFS-Web. The text file, named "3923-40-PCT_ST25", has a size in bytes of 89 KB, and was recorded on 16 August 2012. The information contained in the text file is incorporated herein by reference in its entirety pursuant to 37 CFR § 1.52(e)(5).

FIELD OF THE INVENTION

[0005] The present invention generally relates to yeast-based immunotherapeutic compositions and methods for the prevention and/or treatment of cancers characterized by the expression or overexpression of mucin-1 (MUC1).

BACKGROUND OF THE INVENTION

[0006] Cancer is a leading cause of death worldwide, and the development of effective therapies for cancer continues to be one of the most active areas of research and clinical development. Although a variety of innovative approaches to treat and prevent cancers have been proposed, many cancers continue to have a high rate of mortality and may be difficult to treat or relatively unresponsive to conventional therapies.

[0007] A large number of human carcinomas and hematologic malignancies are characterized, at least in part, by aberrant overexpression of a protein known as mucin-1 (MUC1) whose normal function is to help protect epithelial cells from toxins, microorganisms and other types of external environment stresses (Kufe et al., *Hybridoma* 1984; 3:223-32). MUC1 is a heterodimeric protein formed from the noncovalent interaction of two subunits which are encoded by a single transcript and then processed into subunits post-translationally, known as MUC1-N and MUC1-C. MUC1 is normally found at the apical borders of secretory epithelial cells, and when the cells lose polarity in response to stress, a reversible process for normal cells, MUC1 can interact with molecules that usually localize at the basolateral borders. In addition, in response to stress environments, the MUC1-N subunit, a large protein containing a variable number of tandem repeats (VNTR) that are extensively glycosylated with O-linked glycans, can be shed. The other subunit of MUC1, known as MUC1-C, has an extracellular domain, a transmembrane domain and a cytoplasmic tail, and can bind to a ligand that is responsible for physically associating MUC1 with the epidermal growth factor receptor (EGFR) (Li et al., *J Biol Chem* 2001; 276:35239-42; Schroeder et al., *J Biol Chem* 2001; 276:13057-64) as well as other receptor tyrosine kinases, such as ErbB2-4,20 FGFR321 and PDGFR (Li et al., *Mol Cancer Res* 2003; 1:765-75; Ren et al., *Mol Cancer Res* 2006; 4:873-83; Singh et al., *Cancer Res* 2007; 67:5201-10). In addition, MUC1-C has been associated with a variety of signaling pathways that include ErbB receptors, c-Src, β-catenin, transcription factors (p53, ERα) and other effectors, such as Grb2/SOS (Pandey et al., *Cancer Res* 1995; 55:4000-3; Kinlough et al., *J Biol Chem* 2004; 279:53071-7).

[0008] In transformed epithelial cells, membrane polarity is irreversible and MUC1 expression is upregulated over the entire surface of carcinoma cells (Kufe et al., 1984, *supra*). MUC1 overexpression is associated with decreased MUC1-N O-glycosylation, and the high levels of MUC1-N at the cell surface sterically block cell-cell and cell-extracellular matrix interactions, which are associated with the malignant phenotype (Ligtenberg et al., *Cancer Res* 1992; 52:223-32; van de Wiel-van Kemenade et al., *J Immunol* 1993; 151:767-76; Wesseling et al., *Mol Biol Cell* 1996; 7:565-77). The MUC1-C subunit is now considered to be an oncoprotein, based on its involvement in diverse signaling pathways associated with tumorigenesis, and its overexpression has been shown to be involved in blocking induction of apoptosis in the response to DNA damage (Ren et al., *Cancer Cell* 2004; 5:163-75; Raina et al., *J Biol Chem* 2004; 279:20607-12), oxidative stress (Yin and Kufe, *J Biol Chem* 2003; 278:35458-64; Yin et al., *J Biol Chem* 2004;

279:45721-7), and hypoxia (Yin et al., *J Biol Chem* 2007; 282:257-66), as well as conferring anchorage-independent growth and tumorigenicity (Li et al., *Oncogene* 2003; 22:6107-10; Huang et al., *Cancer Biol Ther* 2003; 2:702-6; Huang et al., *Cancer Res* 2005; 65:10413-22; Schroeder et al., *Oncogene* 2004; 23:5739-47).

[0009] As discussed above, data from various laboratories indicate that the MUC1-N (α subunit) plays a role in cancer by conferring cellular properties that allow immune evasion and potentially metastatic spread. The MUC1-C (β subunit) engages signaling pathways responsible for tumor initiation and progression. These dual functions of MUC1 may explain the differing roles this antigen appears to play in different cancer indications. For example, MUC1 appears to be an early marker in cancers such as breast cancer and colon cancer (*e.g.,* see Kretschmer et al., *Mol Cancer.* 2011 Feb 11;10(1):15; Mukhopadhyay et al., *Biochim Biophys Acta.* 2011 Apr;1815(2):224-40; Saeki et al., *Gastroenterology.* 2011 Mar;140(3):892-902), while MUC1 is associated with epithelial-mesenchymal transition (EMT) pathways and metastatic spread in cancers such as pancreas cancer and esophageal cancer (*e.g.,* see Xu et al., *Life Sci.* 2011 Jun 6;88(23-24):1063-9; Besmer et al., *Cancer Res.* 2011 Jul 1;71(13):4432-42; Roy et al., *Oncogene* 2011 Mar 24;30(12):1449-59; Ye et al., *Lab Invest.* 2011 May;91(5):778-87), and prevents terminal differentiation by reactive oxygen species in acute myeloid leukemia (AML) (*e.g.,* see Yin et al., *Blood.* 2011 May 5;117(18):4863-70; Fatrai et al., *Exp Hematol.* 2008 Oct;36(10):1254-65), thereby allowing unlimited self renewal of these cancer cells.

[0010] Given the apparent role of MUC1 in the malignant phenotype of cancer cells, MUC1, and particularly MUC1-N, has been the focus of anti-cancer therapeutic approaches. Indeed, the majority of therapeutic approaches have targeted MUC1-N, the extracellular portion of the MUC1 heterodimer. However, such approaches targeting MUC1-N have not been successful in the clinic so far, possibly due to interference from MUC1-N that is shed from the cells. More recent studies have proposed targeting the MUC1-C subunit with antibodies against the extracellular domain, or with peptides, peptides conjugated with a carbohydrate polymer, small molecules, with preparations of tumor cells expressing MUC1, and with dendritic cell/tumor cell fusions. However, there are presently no approved cancer therapies that specifically target MUC1. Accordingly, there remains a need in the art for new products that effectively treat and/or prevent cancers associated with MUC1 expression or overexpression.

SUMMARY OF THE INVENTION

[0011] One embodiment of the invention relates to a yeast-MUC1 immunotherapeutic composition, comprising: (a) a yeast vehicle; and (b) a fusion protein expressed by the yeast vehicle and comprising at least one MUC1 antigen. In one aspect, the MUC1 antigen consists of, in order from N- to C-terminus, a MUC1 SEA/extracellular domain (ED), wherein the MUC1 SEA/ED domain comprises a MUC1 ED flanked at the N-terminus by one or more amino acids from the non-ED portion of the MUC1 SEA domain; at least two variable number of tandem repeat (VNTR) domains; a MUC1 transmembrane (TM) domain; and a MUC1 cytoplasmic domain (CD).

[0012] In one aspect, the antigen includes two VNTR domains. In one aspect, the VNTR domain has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to positions 126-145 of SEQ ID NO:11; any consecutive 20 amino acids between positions 61 and 1020 of SEQ ID NO:11; any consecutive 20 amino acids between positions 126 and 965 of SEQ ID NO:11; SEQ ID NO:12; any consecutive 20 amino acids between positions 90 and 130 of SEQ ID NO:14; any consecutive 20 amino acids between positions 60 and 100 of SEQ ID NO:15; and a corresponding sequence from a different human MUC1 protein. In one aspect, the VNTR domain has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to any consecutive 20 amino acids between positions 90 and 130 of SEQ ID NO:14 or any consecutive 20 amino acids between positions 60 and 100 of SEQ ID NO:15. In one aspect, the fusion protein has two VNTR domains, and the amino acid sequence of the two VNTR domains is positions 90 and 130 of SEQ ID NO:14 or positions 60 and 100 of SEQ ID NO:15.

[0013] In one aspect, the MUC1 ED has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from: positions 116-173 of SEQ ID NO:2; positions 107-164 of SEQ ID NO:4; positions 107-164 of SEQ ID NO:6; positions 98-155 of SEQ ID NO:8; positions 1098-1155 of SEQ ID NO:11; positions 32-89 of SEQ ID NO:14; positions 2-59 of SEQ ID NO:15; and a corresponding sequence from a different human MUC1 protein. In one aspect, the MUC1 ED has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to positions 32-89 of SEQ ID NO:14 or positions 2-59 of SEQ ID NO:15. In one aspect, the MUC1 ED has an amino acid sequence of positions 32-89 of SEQ ID NO:14 or positions 2-59 of SEQ ID NO:15. In one aspect, the MUC1 SEA/ED has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid

sequence selected from: positions 115-173 of SEQ ID NO:2; positions 106-164 of SEQ ID NO:4; positions 106-164 of SEQ ID NO:6; positions 97-155 of SEQ ID NO:8; positions 1097-1155 of SEQ ID NO:11; positions 31-89 of SEQ ID NO:14; positions 1-59 of SEQ ID NO:15; and a corresponding sequence from a different human MUC1 protein. In one aspect, the MUC1 SEA/ED has an amino acid sequence of positions 31-89 of SEQ ID NO:14 or positions 1-59 of SEQ ID NO:15.

[0014] In one aspect, the MUC1 TM domain has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from: positions 174-201 of SEQ ID NO:2 , positions 165-192 of SEQ ID NO:4, positions 165-192 of SEQ ID NO:6, positions 156-183 of SEQ ID NO:8, positions 1156-1183 of SEQ ID NO:11, positions 131-158 of SEQ ID NO:14, positions 101-128 of SEQ ID NO:15, and a corresponding sequence from a different human MUC1 protein. In one aspect, the MUC1 TM domain has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to positions 131-158 of SEQ ID NO:14 or positions 101-128 of SEQ ID NO:15. In one aspect, the MUC1 TM domain has an amino acid sequence of positions 131-158 of SEQ ID NO:14 or positions 101-128 of SEQ ID NO:15.

[0015] In one aspect, the MUC1 CD domain has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2, positions 193-264 of SEQ ID NO:4, positions 193-264 of SEQ ID NO:6, positions 184-255 of SEQ ID NO:8, positions 1184-1255 of SEQ ID NO:11, positions 159-230 of SEQ ID NO:14, positions 129-200 of SEQ ID NO:15, positions 7-78 of SEQ ID NO:17, positions 79-150 of SEQ ID NO:17, positions 151-222 of SEQ ID NO:17; positions 1-72 of SEQ ID NO:18, positions 73-144 of SEQ ID NO:18, positions 145-216 of SEQ ID NO:18, and a corresponding sequence from a different human MUC1 protein. In one aspect, the MUC1 CD domain has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence of positions 159-230 of SEQ ID NO:14 or positions 129-200 of SEQ ID NO:15. In one aspect, the MUC1 CD domain has an amino acid sequence of positions 159-230 of SEQ ID NO:14 or positions 129-200 of SEQ ID NO:15.

[0016] In one aspect of this embodiment of the invention, the MUC1 antigen has an amino acid sequence that is at least 95%, 96%, 97%, or 98% identical to SEQ ID NO:15. In one aspect, the MUC1 antigen comprises SEQ ID NO:15 or an amino acid sequence that is at least 99% identical to SEQ ID NO:15. In one aspect, the MUC1 antigen has an amino acid sequence of SEQ ID NO:15.

[0017] In one aspect of this embodiment of the invention, the fusion protein further comprises a MUC1 signal sequence appended to the N-terminus of the MUC1 SEA/ED. In one aspect, the MUC1 signal sequence has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from: positions 1-27 of SEQ ID NO:2, positions 1-32 of SEQ ID NO:4, positions 1-32 of SEQ ID NO:6, positions 1-27 of SEQ ID NO:8, positions 1-23 of SEQ ID NO:11, positions 1-30 of SEQ ID NO:14, and a corresponding sequence from a different human MUC1 protein. In one aspect, the MUC1 signal sequence has an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence of positions 1-30 of SEQ ID NO:14. In one aspect, the MUC1 signal sequence has an amino acid sequence of positions 1-30 of SEQ ID NO:14. In one aspect, the fusion protein has an amino acid sequence that is at least 95%, 96%, 97%, or 98% identical to SEQ ID NO:14. In one aspect, the fusion protein comprises SEQ ID NO:14 or an amino acid sequence that is at least 99% identical to SEQ ID NO:14. In one aspect, the fusion protein has an amino acid sequence of SEQ ID NO:14.

[0018] In one aspect of this embodiment of the invention, the MUC1 antigen comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or more amino acid substitutions, as compared to a wild-type MUC1 sequence, to form between one and 11 agonist epitopes within the MUC1 antigen, also referred to herein as a MUC1 agonist antigen. In one aspect of this embodiment of the invention, the amino acid substitutions are selected from: A96Y, P97L, G104V, S105Y, T106L, A147Y, C161V, T199L, D200F, S215Y, and T239L, with respect to the MUC1 antigen portion of SEQ ID NO:14 or SEQ ID NO:15. In one aspect, the MUC1 agonist antigen has an amino acid sequence that is at least 95%, 96%, 97%, or 98% identical to SEQ ID NO:23. In one aspect, the MUC1 antigen comprises SEQ ID NO:23 or an amino acid sequence that is at least 99% identical to SEQ ID NO:23. In one aspect, the MUC1 antigen has an amino acid sequence of SEQ ID NO:23. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the MUC1 antigen comprises between one and eleven amino acid substitutions to create a MUC1 agonist antigen.

[0019] Another embodiment of the invention relates to a yeast-MUC1 immunotherapeutic composition comprising: (a) a yeast vehicle; and (b) a fusion protein expressed by the yeast vehicle and comprising at least one MUC1 antigen. The MUC1 antigen consists of two or more cytoplasmic domains (CD) of MUC1. In one aspect, the MUC1 antigen consists of three cytoplasmic domains (CD) of MUC1. In one aspect, the three CDs are from the same MUC1 protein. In one aspect, each CD domain comprises an

amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2, positions 193-264 of SEQ ID NO:4, positions 193-264 of SEQ ID NO:6, positions 184-255 of SEQ ID NO:8, positions 1184-1255 of SEQ ID NO:11, positions 159-230 of SEQ ID NO:14, positions 129-200 of SEQ ID NO:15, positions 7-78 of SEQ ID NO:17, positions 79-150 of SEQ ID NO:17, positions 151-222 of SEQ ID NO:17; positions 1-72 of SEQ ID NO:18, positions 73-144 of SEQ ID NO:18, positions 145-216 of SEQ ID NO:18, and a corresponding sequence from a different human MUC1 protein. In one aspect, each CD domain comprises an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from: positions 129-200 of SEQ ID NO:15, positions 7-78 of SEQ ID NO:17, positions 79-150 of SEQ ID NO:17, positions 151-222 of SEQ ID NO:17; positions 1-72 of SEQ ID NO:18, positions 73-144 of SEQ ID NO:18, and positions 145-216 of SEQ ID NO:18.

[0020] In one aspect of this embodiment, the MUC1 antigen has an amino acid sequence that is at least 95%, 96%, 97%, or 98% identical to SEQ ID NO:18. In one aspect, the MUC1 antigen has an amino acid sequence of SEQ ID NO:18 or an amino acid sequence that is at least 99% identical to SEQ ID NO:18. In one aspect, the MUC1 antigen has an amino acid sequence of SEQ ID NO:18. In one aspect, the fusion protein has an amino acid sequence that is at least 95%, 96%, 97%, or 98% identical to SEQ ID NO:17. In one aspect, the fusion protein has an amino acid sequence of SEQ ID NO:17 or an amino acid sequence that is at least 99% identical to SEQ ID NO:17. In one aspect, the fusion protein has an amino acid sequence of SEQ ID NO:17.

[0021] Another embodiment of the invention relates to a yeast-MUC1 immunotherapeutic composition comprising: (a) a yeast vehicle; and (b) a fusion protein expressed by the yeast vehicle and comprising at least one MUC1 agonist antigen. In one aspect of this embodiment of the invention, the MUC1 agonist antigen comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or more amino acid substitutions, as compared to a wild-type MUC1 sequence, to form between one and 11 agonist epitopes within the MUC1 antigen, also referred to herein as a MUC1 agonist antigen. In one aspect, the MUC1 agonist antigen has an amino acid sequence that is at least 95%, 96%, 97%, or 98% identical to SEQ ID NO:23 or SEQ ID NO:25. In one aspect, the MUC1 antigen comprises SEQ ID NO:23 or SEQ ID NO:25 or an amino acid sequence that is at least 99% identical to SEQ ID NO:23 or SEQ ID NO:25. In one aspect, the MUC1 antigen has an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:25.

[0022] Yet another embodiment of the invention relates to a method to reduce tumor burden, inhibit tumor growth, and/or increase survival of an individual who has a cancer that expresses MUC1. The method includes the step of administering to the individual a yeast-MUC1 immunotherapeutic composition described above or elsewhere herein. In one aspect, MUC1 expression is detected in the individual's cancer at the time the composition is first administered. In one aspect, the individual has a stage I cancer. In one aspect, the individual has a stage II cancer. In one aspect, the individual has a stage III cancer. In one aspect, the individual has a stage IV cancer.

[0023] Another embodiment of the invention relates to the use of any of the yeast-MUC1 immunotherapeutic compositions described herein to treat a disease. In one aspect, the disease is cancer.

[0024] Yet another embodiment of the invention relates to the use of any of the yeast-MUC1 immunotherapeutic compositions described herein to reduce, arrest, reverse or prevent the metastatic progression of cancer in an individual who has cancer.

[0025] Yet another embodiment of the invention relates to the use of any of the yeast-MUC1 immunotherapeutic compositions described herein to prevent or delay the onset of a MUC1-expressing cancer.

[0026] Another embodiment of the invention relates to the use of a combination of immunotherapeutic compositions to treat cancer, the immunotherapeutic compositions comprising: (a) a first composition that is any of the yeast-MUC1 immunotherapeutic compositions described herein; and (b) at least one additional immunotherapeutic composition comprising a yeast vehicle and an antigen that is not a MUC1 antigen. In one aspect, the antigen that is not a MUC1 antigen is selected from mutated Ras, carcinoembryonic antigen (CEA), and/or Brachyury.

[0027] In one aspect of any of the embodiments related to a method or use related to a yeast-MUC1 immunotherapy composition described herein, the individual is being treated or has been treated with another therapy for cancer, which can include, but is not limited to, chemotherapy, targeted cancer therapy, radiation therapy, adoptive T cell transfer, surgical resection of a tumor from the individual, and/or the administration of one or more additional immunotherapeutic compositions. In one aspect, the additional immunotherapeutic compositions comprise a second cancer antigen that is a MUC1 antigen or a cancer antigen that is not a MUC1 antigen. In one aspect, the additional immunotherapeutic compositions comprise a yeast vehicle and a second cancer antigen that does not include MUC1 antigen. In one aspect, the additional immunotherapeutic

compositions comprise a second cancer antigen that includes, but is not limited to, mutated Ras, carcinoembryonic antigen (CEA), Brachyury, EGFR, BCR-Abl, MART-1, MAGE-1, MAGE-3, GAGE, GP-100, MUC-2, PSMA, tyrosinase, TRP-1 (gp75), NY-ESO-1, TRP-2, TAG72, KSA, CA-125, PSA, HER-2/neu/c-erb/B2, hTERT, p73, B-RAF, adenomatous polyposis coli (APC), Myc, von Hippel-Lindau protein (VHL), Rb-1, Rb-2, androgen receptor (AR), Smad4, MDR1, Flt-3, BRCA-1, BRCA-2, pax3-fkhr, ews-fli-1, HERV-H, HERV-K, TWIST, Mesothelin, and/or NGEP. In one aspect, the second cancer antigen is selected from the group consisting of: mutated Ras, carcinoembryonic antigen (CEA) and Brachyury. In one aspect, the additional immunotherapeutic composition is a viral vector vaccine. In one aspect, the additional immunotherapeutic composition is a dendritic cell/tumor cell fusion.

[0028] Yet another embodiment of the invention relates to a method to prevent or delay the onset of a MUC1-expressing cancer. The method includes a step of administering to an individual any of the yeast-MUC1 immunotherapeutic compositions described herein. In one aspect, cancer has not been detected in the individual. In one aspect, the individual is at high risk for developing cancer. In one aspect, the individual has a pre-cancerous lesion. In one aspect, the individual has cancer, but MUC1-expressing cancer cells have not been detected in the cancer.

[0029] In any of the methods or uses related to a yeast-MUC1 immunotherapy composition described herein, in one aspect, the cancer is of epithelial cell origin. In one aspect, the cancer can include, but is not limited to: breast cancer, small intestine cancer, stomach cancer, pancreatic cancer, kidney cancer, bladder cancer, uterine cancer, ovarian cancer, testicular cancer, lung cancer, colon cancer, prostate cancer, melanoma, multiple myelogenous leukemia (MML), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), Burkitt's lymphoma, Hodgkin's lymphoma, cancers of secretory tissues, and metastatic cancers thereof. In one aspect, the cancer is selected from breast cancer and colon cancer. In one aspect, the cancer is selected from breast cancer, colon cancer, pancreas cancer, ovarian cancer, esophageal cancer, and AML. In one aspect, the cancer is AML, and the yeast-MUC1 immunotherapeutic composition is administered to both donor and recipient of bone marrow transplantation (BMT) therapy. In one aspect, the cancer is AML, and the yeast-MUC1 immunotherapeutic composition is administered to the individual in conjunction with cytarabine and anthracycline therapy.

[0030] In any of the embodiments of the invention described above or elsewhere herein, in one aspect, the yeast vehicle is a whole yeast. In one aspect, the yeast vehicle is

heat-inactivated. In one aspect, the yeast vehicle is from a mutant yeast strain that produces underglycosylated proteins, as compared to a wild-type yeast strain. In one aspect, the MUC1 antigen is expressed on the cell wall of the yeast vehicle. In one aspect, the MUC1 antigen is expressed in the periplasm or cytoplasm of the yeast vehicle. In one aspect, the yeast vehicle is from *Saccharomyces*. In one aspect, the yeast vehicle is from *Saccharomyces cerevisiae*.

[0031] In any of the embodiments of the invention described above or elsewhere herein, in one aspect, the immunotherapeutic composition has been produced by culturing a whole yeast expressing the MUC1 antigen in a medium that was maintained at a pH level of between 5.5 and 8. In one aspect, the medium was buffered with a buffering agent. In one aspect, the yeast was cultured in a medium that was maintained at a pH level of between 6 and 8.

[0032] In any of the embodiments of the invention described above or elsewhere herein, in one aspect, the composition further comprises at least one biological response modifier.

[0033] In any of the embodiments of the invention described above or elsewhere herein, in one aspect, the composition further comprises a pharmaceutically acceptable excipient.

[0034] In any of the embodiments of the invention described above or elsewhere herein, in one aspect, the composition has been formulated for injection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] Fig. 1A is a schematic drawing showing the structure of full-length MUC1 protein.

[0036] Fig. 1B is a schematic drawing showing the structure of the fusion protein expressed in the yeast-based immunotherapeutic composition known as GI-6101.

[0037] Fig. 1C is a schematic drawing showing the structure of the fusion protein expressed in the yeast-based immunotherapeutic composition known as GI-6104.

[0038] Fig. 2A is a digitized image showing expression of MUC1 fusion protein by GI-6101.

[0039] Fig. 2B is a digitized image showing expression of MUC1 fusion proteins from GI-6101 and 6104 before and after deglycosylation.

[0040] Fig. 2C is a digitized image showing expression of MUC1 fusion protein by GI-6104.

[0041] Fig. 3 is a digitized image showing expression of MUC1 fusion protein by GI-6105.

DETAILED DESCRIPTION OF THE INVENTION

[0042] This invention generally relates to yeast-based immunotherapeutic compositions and methods for the prevention and/or treatment of cancers that express or overexpress mucin-1 (which may generally be referred to herein as "MUC1", and which is also known or has been known as "DF3 antigen" or "HMFG"). The invention includes the use of a yeast-based immunotherapeutic composition (also referred to as yeast-based immunotherapy composition or product) comprising a yeast vehicle and novel MUC1 antigens (also referred to herein as "yeast-MUC1 immunotherapy composition", "yeast-MUC1 immunotherapy product" or "yeast-MUC1 immunotherapeutic compositions"). The inventors describe herein the construction and production of novel yeast-MUC1 immunotherapy products, and demonstrate that yeast-MUC1 immunotherapy matures human dendritic cells (DCs), increases cytokine production from DCs that is associated with immune responses expected to be beneficial in the treatment of tumors, and elicits the activation of MUC1-specific T cell lines. Taken together, the data presented herein show that yeast-MUC1 immunotherapy is useful for the elicitation of MUC1-specific cellular immune responses ($CD4^+$ and $CD8^+$) and that yeast-MUC1 immunotherapy is expected to be useful for the prevention and treatment of MUC1-expressing tumors.

[0043] Yeast-MUC1 immunotherapy is readily adaptable to the use of additional tumor antigens within the same yeast composition, or to use in combination with other yeast-based immunotherapeutics that target other tumor antigens (sequentially or concurrently) or other immunotherapeutics and treatments/therapies for cancer. Accordingly, the Yeast-MUC1 immunotherapy can be adapted to the cancer type, the cancer stage, the cancer grade, the antigens expressed by the tumor, and the overall medical status of the individual (*i.e.,* the therapy is easily personalized), and for the individual who already has cancer, its use can be modified as cancer progresses in an individual, in order to provide maximum efficacy at a variety of tumor stages. Yeast-MUC1 immunotherapy offers the opportunity for the broad-based prophylactic and/or therapeutic treatment of a wide range of cancers.

[0044] Indeed, yeast-MUC1 immunotherapy can be used in a flexible manner to treat various MUC1-positive cancers by tailoring the yeast-MUC1 immunotherapy to the particular role this antigen plays in each type of cancer indication. For example, since

MUC1 has been described as an early marker in cancers such as breast cancer or colon cancer, yeast-based immunotherapy may be used prophylactically in patients with MUC1 positive premalignant breast hyperplasia or colonic polyps. As another example, since MUC1 has been associated with epithelial-mesenchymal transition (EMT) pathways and metastatic spread in cancers such as pancreas cancer, ovarian cancer, and esophageal cancer, yeast-MUC1 immunotherapy can be used as a therapeutic add-on to standard of care therapy in these cancers to promote the arrest of metastatic spread in MUC1-positive stage 3 pancreas, ovarian, and esophageal cancers. As yet another example, since MUC1 has been shown to prevent terminal differentiation by reactive oxygen species in acute myeloid leukemia (AML), thereby allowing unlimited self renewal of these cancer cells, yeast-MUC1 immunotherapy can be used as an add-on to standard therapy in MUC1-positive AML to promote apoptosis and prevent the unlimited self renewal of the malignant cells. A clinical trial using yeast-MUC1 immunotherapy in AML patients is described in the examples.

[0045] Yeast-MUC1 compositions described herein induce innate immune responses, as well as adaptive immune responses against the target antigen (MUC1), including CD4-dependent TH17 and TH1 T cell responses and antigen-specific $CD8^+$ T cell responses, which include cytotoxic T lymphocyte (CTL) responses, all without the use of exogenous adjuvants, cytokines, or other immunostimulatory molecules, many of which have to toxicity issues. In addition, Yeast-MUC1 immunotherapeutic compositions inhibit regulatory T cell (Treg) numbers and/or functionality, thereby enhancing effector T cell responses that might normally be suppressed by the presence of the tumor, for example. Moreover, as compared to immunotherapeutic compositions that immunize by generating antibody responses, the antigen-specific, broad-based, and potent cellular immune responses elicited by Yeast-MUC1 immunotherapy are believed to be particularly effective in targeting tumor cells. Indeed, numerous studies have shown that immunotherapeutic approaches are enhanced when tumor cells are targeted via $CD8^+$ CTLs which recognize tumor peptides in the context of MHC Class I molecules. Yeast-MUC1 immunotherapy is highly adept at activating antigen presenting cells, and has a unique ability to cross-prime the immune response, generating $CD8^+$ CTL responses that are typically effective against tumors, even in the face of what may otherwise be a suppressive environment. Since this type of immunotherapy utilizes the natural ability of the antigen presenting cell to present relevant immunogens, it is not necessary to know the precise identity of CTL epitopes or MHC Class II epitopes of MUC1 to produce an

effective immunotherapeutic according to the present invention. In fact, multiple $CD4^+$ and $CD8^+$ T cell epitopes can be targeted in a single Yeast-MUC1 immunotherapeutic composition, and so the Yeast-MUC1 immunotherapeutics of the invention are not limited to the use of short peptides. Indeed, the use of longer polypeptides and fusion proteins containing multiple domains of the target antigen in these compositions is efficacious. Accordingly, by using Yeast-MUC1 immunotherapy, the use of algorithms and complex formulas to identify putative T cell epitopes is eliminated.

[0046] Yeast-MUC1 can be effectively utilized in an immunization protocol (prophylactic or therapeutic) without the use of exogenous adjuvants, immunostimulatory agents or molecules, costimulatory molecules, or cytokines, although such agents may be included, if desired. Moreover, Yeast-MUC1 immunotherapy can be administered repeatedly without losing efficacy, as may be problematic with other types of immunotherapy.

Compositions of the Invention

[0047] One embodiment of the present invention relates to a yeast-based immunotherapy composition which can be used to prevent and/or treat cancers characterized by MUC1 expression or overexpression (including cancers that may not contain cells expressing detectable MUC1 initially, but which may or will contain cells expressing MUC1 at later stages of the development of the cancer). The composition is a Yeast-MUC1 immunotherapeutic composition comprising: (a) a yeast vehicle; and (b) a cancer antigen comprising one or more MUC1 antigen(s) and/or immunogenic domain(s) thereof. The MUC1 antigen or immunogenic domain thereof is most typically expressed as a recombinant protein by the yeast vehicle (*e.g.,* by an intact yeast or yeast spheroplast, which can optionally be further processed to a yeast cytoplast, yeast ghost, or yeast membrane extract or fraction thereof), although it is an embodiment of the invention that one or more MUC1 antigens are loaded into a yeast vehicle or otherwise complexed with, attached to, mixed with or administered with a yeast vehicle as described herein to form a composition of the present invention.

[0048] A "Yeast-MUC1 immunotherapeutic composition" is a specific type of "yeast-based immunotherapeutic composition" that contains at least one MUC1 antigen or immunogenic domain thereof. The phrase, "yeast-based immunotherapeutic composition" may be used interchangeably with "yeast-based immunotherapy product", "yeast-based immunotherapy composition", "yeast-based composition", "yeast-based immunotherapeutic", "yeast-based vaccine", or derivatives of these phrases. An

"immunotherapeutic composition" is a composition that elicits an immune response sufficient to achieve at least one therapeutic benefit in a subject. As used herein, yeast-based immunotherapeutic composition refers to a composition that includes a yeast vehicle component and that elicits an immune response sufficient to achieve at least one therapeutic benefit in a subject. More particularly, a yeast-based immunotherapeutic composition is a composition that includes a yeast vehicle component and typically, an antigen component, and can elicit or induce an immune response, such as a cellular immune response, including without limitation a T cell-mediated cellular immune response. In one aspect, a yeast-based immunotherapeutic composition useful in the invention is capable of inducing a $CD8^+$ and/or a $CD4^+$ T cell-mediated immune response and in one aspect, a $CD8^+$ and a $CD4^+$ T cell-mediated immune response, particularly against a target antigen (*e.g.,* a cancer antigen). A $CD4^+$ immune response can include TH1 immune responses, TH2 immune responses, TH17 immune responses, or any combination of the above. Yeast-based immunotherapeutics are particularly capable of generating TH1 and TH17 responses. A $CD8^+$ immune response can include a cytotoxic T lymphocyte (CTL) response, and yeast-based immunotherapeutics are capable of generating such responses. In one aspect, a yeast-based immunotherapeutic composition modulates the number and/or functionality of regulatory T cells (Tregs) in a subject. Yeast-based immunotherapy can also be modified to promote one type of response over another, *e.g.,* by the addition of cytokines, antibodies, and/or modulating the manufacturing process for the yeast. Optionally, a yeast-based immunotherapeutic composition is capable of eliciting a humoral immune response.

[0049] Yeast-MUC1 immunotherapeutic compositions of the invention may be either "prophylactic" or "therapeutic". When provided prophylactically, the compositions of the present invention are provided in advance of the development of, or the detection of the development of, a cancer that expresses MUC1, with the goal of preventing, inhibiting or delaying the development of MUC1-expressing tumors; and/or preventing, inhibiting or delaying metastases of such tumors and/or generally preventing or inhibiting progression of cancer in an individual. As discussed herein, MUC1 is expressed in several cancers. Therefore, prophylactic compositions can be administered to individuals that appear to be cancer-free (healthy, or normal, individuals), to individuals with pre-cancerous (pre-malignant lesions), and also to individuals who have cancer, but in which MUC1 has not yet been detected (*i.e.* prior to the expression of MUC1 by tumor cells in the cancer). Individuals who are at high risk for developing a cancer, particularly a cancer with which

MUC1 expression and/or metastases are typically associated, may be treated prophylactically with a composition of the invention. When provided therapeutically, the immunotherapy compositions are provided to an individual with a MUC1-expressing cancer, with the goal of ameliorating the cancer, such as by reducing tumor burden in the individual; inhibiting tumor growth in the individual; increasing survival of the individual; and/or preventing, inhibiting, reversing or delaying progression of the cancer in the individual.

[0050] Typically, a Yeast-MUC1 immunotherapy composition includes a yeast vehicle and at least one cancer antigen comprising a MUC1 antigen or immunogenic domain thereof, where the cancer antigen is expressed by, attached to, loaded into, or mixed with the yeast vehicle. In some embodiments, the cancer antigen, MUC1 antigen, or immunogenic domain thereof is provided as a fusion protein. Several MUC1 proteins and fusion proteins suitable for use in the compositions and methods of the invention are described below. In some embodiments, the cancer antigen and the MUC1 antigen are the same element. In some embodiments, the cancer antigen includes other antigens, including other cancer antigens, in addition to the MUC1 antigen. In one aspect of the invention, a fusion protein useful as a cancer antigen can include two or more antigens, *e.g.,* a MUC1 antigen and another cancer antigen that is not a MUC1 antigen, or two different MUC1 antigens. In one aspect, the fusion protein can include two or more immunogenic domains of one or more antigens, such as two or more immunogenic domains of a MUC1 antigen, or two or more epitopes of one or more antigens, such as two or more epitopes of a MUC1 antigen.

[0051] According to the present invention, a yeast vehicle used in a Yeast-MUC1 immunotherapy composition is any yeast cell (*e.g.,* a whole or intact cell) or a derivative thereof (see below) that can be used in conjunction with one or more antigens, immunogenic domains thereof or epitopes thereof in a composition of the invention (*e.g.,* a therapeutic or prophylactic composition). The yeast vehicle can therefore include, but is not limited to, a live intact (whole) yeast microorganism (*i.e.,* a yeast cell having all its components including a cell wall), a killed (dead) or inactivated intact yeast microorganism, or derivatives of intact yeast including: a yeast spheroplast (*i.e.,* a yeast cell lacking a cell wall), a yeast cytoplast (*i.e.*, a yeast cell lacking a cell wall and nucleus), a yeast ghost (*i.e.,* a yeast cell lacking a cell wall, nucleus and cytoplasm), a subcellular yeast membrane extract or fraction thereof (also referred to as a yeast membrane particle

and previously as a subcellular yeast particle), any other yeast particle, or a yeast cell wall preparation.

[0052] Yeast spheroplasts are typically produced by enzymatic digestion of the yeast cell wall. Such a method is described, for example, in Franzusoff et al., 1991, *Meth. Enzymol.* 194, 662-674., incorporated herein by reference in its entirety.

[0053] Yeast cytoplasts are typically produced by enucleation of yeast cells. Such a method is described, for example, in Coon, 1978, *Natl. Cancer Inst. Monogr.* 48, 45-55 incorporated herein by reference in its entirety.

[0054] Yeast ghosts are typically produced by resealing a permeabilized or lysed cell and can, but need not, contain at least some of the organelles of that cell. Such a method is described, for example, in Franzusoff et al., 1983, *J. Biol. Chem.* 258, 3608-3614 and Bussey et al., 1979, *Biochim. Biophys. Acta* 553, 185-196, each of which is incorporated herein by reference in its entirety.

[0055] A yeast membrane particle (subcellular yeast membrane extract or fraction thereof) refers to a yeast membrane that lacks a natural nucleus or cytoplasm. The particle can be of any size, including sizes ranging from the size of a natural yeast membrane to microparticles produced by sonication or other membrane disruption methods known to those skilled in the art, followed by resealing. A method for producing subcellular yeast membrane extracts is described, for example, in Franzusoff et al., 1991, *Meth. Enzymol.* 194, 662-674. One may also use fractions of yeast membrane particles that contain yeast membrane portions and, when the antigen or other protein was expressed recombinantly by the yeast prior to preparation of the yeast membrane particles, the antigen or other protein of interest. Antigens or other proteins of interest can be carried inside the membrane, on either surface of the membrane, or combinations thereof (*i.e.,* the protein can be both inside and outside the membrane and/or spanning the membrane of the yeast membrane particle). In one embodiment, a yeast membrane particle is a recombinant yeast membrane particle that can be an intact, disrupted, or disrupted and resealed yeast membrane that includes at least one desired antigen or other protein of interest on the surface of the membrane or at least partially embedded within the membrane.

[0056] An example of a yeast cell wall preparation is a preparation of isolated yeast cell walls carrying an antigen on its surface or at least partially embedded within the cell wall such that the yeast cell wall preparation, when administered to an animal, stimulates a desired immune response against a disease target.

[0057] Any yeast strain can be used to produce a yeast vehicle of the present invention. Yeast are unicellular microorganisms that belong to one of three classes: Ascomycetes, Basidiomycetes and Fungi Imperfecti. One consideration for the selection of a type of yeast for use as an immune modulator is the pathogenicity of the yeast. In one embodiment, the yeast is a non-pathogenic strain such as *Saccharomyces cerevisiae*. The selection of a non-pathogenic yeast strain minimizes any adverse effects to the individual to whom the yeast vehicle is administered. However, pathogenic yeast may be used if the pathogenicity of the yeast can be negated by any means known to one of skill in the art (*e.g.,* mutant strains). In accordance with one aspect of the present invention, non-pathogenic yeast strains are used.

[0058] Genera of yeast strains that may be used in the invention include but are not limited to *Saccharomyces, Candida* (which can be pathogenic), *Cryptococcus, Hansenula, Kluyveromyces, Pichia, Rhodotorula, Schizosaccharomyces* and *Yarrowia*. In one aspect, yeast genera are selected from *Saccharomyces, Candida, Hansenula, Pichia* or *Schizosaccharomyces*, and in one aspect, *Saccharomyces* is used. Species of yeast strains that may be used in the invention include but are not limited *to Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Candida albicans, Candida kefyr, Candida tropicalis, Cryptococcus laurentii, Cryptococcus neoformans, Hansenula anomala, Hansenula polymorpha, Kluyveromyces fragilis, Kluyveromyces lactis, Kluyveromyces marxianus var. lactis, Pichia pastoris, Rhodotorula rubra, Schizosaccharomyces pombe,* and *Yarrowia lipolytica*. It is to be appreciated that a number of these species include a variety of subspecies, types, subtypes, etc. that are intended to be included within the aforementioned species. In one aspect, yeast species used in the invention include *S. cerevisiae, C. albicans, H. polymorpha, P. pastoris* and *S. pombe*. *S. cerevisiae* is useful as it is relatively easy to manipulate and being "Generally Recognized As Safe" or "GRAS" for use as food additives (GRAS, FDA proposed Rule 62FR18938, April 17, 1997). One embodiment of the present invention is a yeast strain that is capable of replicating plasmids to a particularly high copy number, such as a *S. cerevisiae* cir° strain. The *S. cerevisiae* strain is one such strain that is capable of supporting expression vectors that allow one or more target antigen(s) and/or antigen fusion protein(s) and/or other proteins to be expressed at high levels. Another yeast strain is useful in the invention is *Saccharomyces cerevisiae* W303α. In addition, any mutant yeast strains can be used in the present invention, including those that exhibit reduced post-translational modifications of expressed target antigens or other proteins, such as mutations in the enzymes that extend

N-linked glycosylation. In one aspect of the invention, a yeast-MUC1 immunotherapy composition is produced using a mutant yeast strain that produces the MUC1 antigen as an underglycosylated protein as compared to the same antigen produced by the wild-type strain (with normal glycosylation). Such a MUC1 antigen may be more similar to MU1 antigens expressed by tumor cells, which can then be processed into unique T cell epitopes by antigen presenting cells, thus enhancing the specific anti-tumor response.

[0059] The Yeast-MUC1 immunotherapy composition of the invention includes at least one cancer antigen comprising a MUC1 antigen. According to the present invention, the general use herein of the term "antigen" refers: to any portion of a protein (*e.g.,* peptide, partial protein, full-length protein), wherein the protein is naturally occurring or synthetically derived or designed, to a cellular composition (whole cell, cell lysate or disrupted cells), to an organism (whole organism, lysate or disrupted cells) or to a carbohydrate, or other molecule, or a portion thereof. An antigen may elicit an antigen-specific immune response (*e.g.,* a humoral and/or a cell-mediated immune response) against the same or similar antigens that are encountered *in vitro, in vivo,* or *ex vivo* by an element of the immune system (*e.g.,* T cells, antibodies).

[0060] An antigen can be as small as a single epitope, a single immunogenic domain or larger, and can include multiple epitopes or immunogenic domains. As such, the size of an antigen can be as small as about 8-11 amino acids (*i.e.,* a peptide) and as large as: a domain of a protein, a full-length protein, a multimer, a fusion protein, a chimeric protein, a whole cell, a whole microorganism, or any portions thereof (*e.g.,* protein fragments (polypeptides) lysates of whole cells or extracts of microorganisms). Antigens useful in the Yeast-MUC1 immunotherapeutic of the present invention are peptides, polypeptides, protein domain(s), protein subunits, full-length proteins, multimers, fusion proteins and chimeric proteins. In addition, antigens can include carbohydrates, which can be loaded into a yeast vehicle or into a composition of the invention. It will be appreciated that in some embodiments (*e.g.,* when the antigen is expressed by the yeast vehicle from a recombinant nucleic acid molecule), the antigen is a protein (including fragments, domains, subunits, and full-length proteins), fusion protein, chimeric protein, or fragment thereof, rather than an entire cell or microorganism. For expression in yeast, in one embodiment, an antigen is of a minimum size capable of being expressed recombinantly in yeast if the antigen is the entire protein to be expressed by the yeast (in other words, the protein that is expressed by the yeast, which may include or consist of the antigen, is preferably at least 25 amino acids in length), and is typically at least or greater than 25 amino acids in length,

or at least or greater than 26 amino acids, at least or greater than 27 amino acids, at least or greater than 28 amino acids, at least or greater than 29 amino acids, at least or greater than 30 amino acids, at least or greater than 31 amino acids, at least or greater than 32 amino acids, at least or greater than 33 amino acids, at least or greater than 34 amino acids, at least or greater than 35 amino acids, at least or greater than 36 amino acids, at least or greater than 37 amino acids, at least or greater than 38 amino acids, at least or greater than 39 amino acids, at least or greater than 40 amino acids, at least or greater than 41 amino acids, at least or greater than 42 amino acids, at least or greater than 43 amino acids, at least or greater than 44 amino acids, at least or greater than 45 amino acids, at least or greater than 46 amino acids, at least or greater than 47 amino acids, at least or greater than 48 amino acids, at least or greater than 49 amino acids, or at least or greater than 50 amino acids in length, or at least 25-50 amino acids in length, at least 30-50 amino acids in length, or at least 35-50 amino acids in length, or at least 40-50 amino acids in length, or at least 45-50 amino acids in length, although smaller proteins may be expressed, and considerably larger proteins (*e.g.,* hundreds of amino acids in length or even a few thousand amino acids in length) may be expressed. In one aspect, a full-length protein or domain of a protein that is lacking between 1 and 20 amino acids from the N- and/or the C-terminus may be expressed. Fusion proteins and chimeric proteins are also antigens that may be expressed in the invention. A "target antigen" is an antigen that is specifically targeted by an immunotherapeutic composition of the invention (*i.e.,* an antigen, usually the native antigen, against which elicitation of an immune response is desired). A "cancer antigen" is an antigen that comprises at least one antigen that is associated with a cancer such as an antigen expressed by a tumor cell, so that targeting the antigen also targets the tumor cell and/or cancer. A cancer antigen can include one or more antigens from one or more proteins, including one or more tumor-associated proteins. A "MUC1 antigen" is an antigen derived, designed, or produced from a MUC1 protein (including MUC1-N, MUC1-C or both MUC1-N and MUC1-C).

[0061] When referring to stimulation of an immune response, the term "immunogen" is a subset of the term "antigen", and therefore, in some instances, can be used interchangeably with the term "antigen". An immunogen, as used herein, describes an antigen which elicits a humoral and/or cell-mediated immune response (*i.e.,* is immunogenic), such that administration of the immunogen to an individual mounts an antigen-specific immune response against the same or similar antigens that are encountered by the immune system of the individual. In one embodiment, the immunogen

elicits a cell-mediated immune response, including a $CD4^+$ T cell response (*e.g.,* TH1, TH2 and/or TH17) and/or a $CD8^+$ T cell response (*e.g.,* a CTL response).

[0062] An "immunogenic domain" or "immunological domain" of a given antigen can be any portion, fragment or epitope of an antigen (*e.g.*, a peptide fragment or subunit or an antibody epitope or other conformational epitope) that contains at least one epitope that can act as an immunogen when administered to an animal. Therefore, an immunogenic domain is larger than a single amino acid and is at least of a size sufficient to contain at least one epitope that can act as an immunogen. For example, a single protein can contain multiple different immunogenic domains. Immunogenic domains need not be linear sequences within a protein, such as in the case of a humoral immune response, where conformational domains are contemplated.

[0063] An epitope is defined herein as a single immunogenic site within a given antigen that is sufficient to elicit an immune response when provided to the immune system in the context of appropriate costimulatory signals and/or activated cells of the immune system. In other words, an epitope is the part of an antigen that is recognized by components of the immune system, and may also be referred to as an antigenic determinant. Those of skill in the art will recognize that T cell epitopes are different in size and composition from B cell or antibody epitopes, and that epitopes presented through the Class I MHC pathway differ in size and structural attributes from epitopes presented through the Class II MHC pathway. For example, T cell epitopes presented by Class I MHC molecules are typically between 8 and 11 amino acids in length, whereas epitopes presented by Class II MHC molecules are less restricted in length and may be up to 25 amino acids or longer. In addition, T cell epitopes have predicted structural characteristics depending on the specific MHC molecules bound by the epitope. Epitopes can be linear sequence epitopes or conformational epitopes (conserved binding regions). Most antibodies recognize conformational epitopes.

[0064] MUC1 (which may also be referred to as "mucin-1" and also "DF3 antigen" or "HMFG1") is a large glycoprotein expressed by most epithelial secretory tissues at basal levels and is expressed at high levels by malignancies of epithelial cell origin. MUC1 is most typically found as a polymorphic, type I transmembrane protein with a large extracellular domain (also referred to as MUC1-N subunit) that includes variable numbers of tandem repeats (VNTR; typically between 20 and 125 repeats) that are highly glycosylated through O-linkages. The MUC1 protein is encoded as a single transcript, and then processed into subunits post-translationally, known as MUC1-N and MUC1-C, or α

and β subunits, respectively, which then form a heterodimeric protein by a strong noncovalent interaction of the two subunits. MUC1 is cleaved into its N- and C-subunits within the "sea urchin sperm protein, enterokinase and agrin" (SEA) domain, a highly conserved protein domain that was named based on its initial identification in a sperm protein, in enterokinase, and in agrin, and that is found in a number of heavily glycosylated mucin-like proteins that are typically membrane-tethered. The MUC1 protein is cleaved between glycine and serine residues present in the sequence GSVVV (*e.g.,* positions 1097-1101 of SEQ ID NO:11) within the SEA domain (Lillehoj et al., 2003, *Biochem. Biophys. Res. Commun.* 307:743–749; Parry et al., 2001, *Biochem. Biophys. Res. Commun.* 283:715–720; Wreschner et al., 2002, *Protein Sci.* 11:698–706).

[0065] The MUC1-C subunit includes the extracellular domain (ED), which is glycosylated and binds the galectin-3 ligand, which in turn serves as a bridge to physically associate MUC1 with the epidermal growth factor receptor (EGFR) and possibly other receptor tyrosine kinases. MUC1-C also comprises a transmembrane (TM) domain, and a cytoplasmic domain (CD) which contains several tyrosine residues which, when phosphorylated, could act as binding motifs for proteins with SH2 domains (for a detailed discussion of the MUC1 protein and known and putative functions, see Kufe, 2008, Cancer Biol. & Ther. 7:81-84). Alternative splice variants of MUC1 (known as MUC1/Y and MUC1/X, for example) are "short" versions of MUC1 that lack most of MUC1-N, including the large VNTR region, but that include the ED, TM and CD regions, as well as the SEA domain and portions of the N-terminal region signal sequence region. Cleavage within the SEA domain may not occur in these short versions.

[0066] The isolation and sequencing of DNA and cDNA encoding human MUC1 has been reported (see, *e.g.,* Siddiqui et al., 1998, *PNAS* 85:2320-2323; Abe and Kufe, 1993, *PNAS* 90:282-286; Hareuveni et al., 1990, *Eur.J. Biochem.* 189(3) 475-486; Gendler et al., 1990, *J. Biol. Chem.* 265 (25) 15286-15293; Lan et al., 1990, *J. Biol. Chem* 265(25) 15294-15299; Tsarfaty et al., 1990, *Gene* 93(2) 313-318; Lancaster, 1990, *Biochem. Biophys. Res. Commun.* 173(3) 1019-1029). An example of a full-length human MUC1 precursor protein containing both the MUC1-N and MUC1-C regions is described in SwissProt Accession No. P15941.3 (GI:296439295), and is represented here by SEQ ID NO:11. 10 different MUC1 isoforms can be created from the gene encoding SEQ ID NO:11 by alternative transcript splicing. For example, an isoform known as MUC1/Y lacks positions 54-1053 of SEQ ID NO:11. Various other isoforms are described in the database description of this protein.

[0067] For purposes of illustration of the identification of domains within the MUC1 protein, which can be applied to any human MUC1 protein as well as other mammalian MUC1 proteins, the following domains can be readily identified in SEQ ID NO:11. The MUC1 signal sequence, also referred to herein as the leader sequence, is located at about positions 1-23 of SEQ ID NO:11 (the MUC1 signal sequence is identified as longer in some MUC1 variants, and may include additional amino acids, such as positions 1-32). The MUC1-N subunit or α subunit comprises approximately positions 24-1097 of SEQ ID NO:11, and the MUC1-C subunit or β subunit comprises approximately positions 1098-1255 of SEQ ID NO:11.

[0068] Within the MUC1-N subunit, the VNTR (variable numbers of tandem repeats) domain can be found, comprising multiple repeats in this particular protein, including approximately the region from position 126-965, which contains forty-two 20-amino acid repeats of the sequence PAPGSTAPPAHGVTSAPDTR (*e.g.,* positions 126-145 of SEQ ID NO:11), which is the commonly recognized VNTR sequence (see also SEQ ID NO:12 below, which designates common polymorphisms within this sequence). Since these are repeated sequences, one may begin counting from any one of the 20 amino acids in one VNTR and then restart numbering with the repeat of that first amino acid. More particularly, since a single VNTR domain is an approximately 20 amino acid sequence that is preceded by and/or followed by another identical, nearly identical, or homologous 20 amino acid sequence, which may be within a large number of such repeated sequences, for purposes of describing a single VNTR within a region of VNTRs, one may consider "position 1" of a given VNTR to be any one of the 20 amino acids in the VNTR, and then the prior and subsequent flanking amino acids will be numbered accordingly, with the amino acid that is upstream of (prior to) position 1 being either the last amino acid (position 20) of the prior VNTR or the last amino acid of the sequence linked to the VNTR (if such prior sequence is not also a VNTR), and the amino acid that is downstream of position 1 being position 2 of that VNTR, followed by position 3, and so on, until the sequence repeats with the next VNTR.

[0069] Positions 61-1120 of SEQ ID NO:11 includes the VNTR region discussed above, plus additional regions denoted as "repetitive regions". For example, positions 81-100, positions 101-120, positions 121-140, positions 141-160, positions 161-180, positions 181-200, positions 201-220, positions 221-240, positions 241-260, positions 261-180, positions 281-300, and so on, in 20 amino acid increments through positions 1001-1120 of SEQ ID NO:11, represent repetitive regions in this protein.

[0070] In the full-length MUC1 protein represented by SEQ ID NO:11 (prior to cleavage into subunits), the SEA domain spans positions 1034-1152 of SEQ ID NO:11. Cleavage of the SEA domain between amino acids 1097 and 1098 of SEQ ID NO:11 produces the MUC1-C domain. Within the MUC1-C domain, the extracellular domain (ED) is found at about positions 1098-1155 of SEQ ID NO:11; the transmembrane (TM) domain is found at about positions 1156-1183 of SEQ ID NO:11; and the cytoplasmic domain (CD, also called the cytoplasmic tail) is found at about positions 1184-1255 of SEQ ID NO:11.

[0071] The number of VNTR in a given MUC1-N subunit is highly polymorphic, and can vary, *e.g.,* from 20 to 125 repeats. The tandemly repeated icosapeptide typically has a polymorphism at one or more of three positions (positions 9, 18 and 19 of SEQ ID NO:12): PAPGSTAP[P/A/Q/T]AHGVTSAP[DT/ES]R (SEQ ID NO:12, bracketed regions indicate common polymorphisms), where the polymorphism at positions 18 and 19 of SEQ ID NO:12 occur with the preference of DT > ES, and where the single replacements at position 9 occur with the following preference: P > A, P > Q and P > T. The most frequent replacement, DT > ES, occurs in up to 50% of the repeats.

[0072] A variety of transcript variants of MUC1 are known, but the MUC1 subunits, domains, or regions described in the exemplary SEQ ID NO:11 above can readily be identified in the variants, such that a MUC1 antigen useful in the invention can be designed or produced based on a given MUC1 sequence, or a corresponding sequence from another MUC1 protein. For example, one nucleotide sequence encoding a human MUC1 protein is represented herein by SEQ ID NO:1, which corresponds to GENBANK® Accession No. NM_002456.4 (GI: 65301116). SEQ ID NO:1 encodes a 273 amino acid human MUC1 protein (transcript variant 1, also known as MUC1/ZD), the amino acid sequence of which is represented here as SEQ ID NO:2 (also found in GENBANK® Accession No. NP_002447.4; GI:65301117). Within SEQ ID NO:2, the following domains are present: signal sequence (positions 1-27 of SEQ ID NO:2); SEA domain (positions 55-170 of SEQ ID NO:2); ED (positions 116-173 of SEQ ID NO:2); TM domain (positions 174-201 of SEQ ID NO:2); and CD (positions 202-273 of SEQ ID NO:2). The proteolytic cleavage site within the SEA domain that cleaves the ED domain from the N-terminal portion of the SEA domain is between positions 115 and 116 of SEQ ID NO:2. This transcript variant does not contain the VNTR region as shown in SEQ ID NO:11.

[0073] Another nucleotide sequence encoding another human MUC1 protein is represented herein by SEQ ID NO:3, which corresponds to GENBANK® Accession No. NM_001018016.1 (GI:67189006). SEQ ID NO:3 encodes a 264 amino acid human MUC1 protein (transcript variant 2, also known as "MUC1/Y"), the amino acid sequence of which is represented here as SEQ ID NO:4 (also found in GENBANK® Accession No. NP_001018016.1; GI:67189007). Within SEQ ID NO:4, the following domains are present: signal sequence (positions 1-32 of SEQ ID NO:4); SEA domain (positions 45-161 of SEQ ID NO:4); ED (107-164 of SEQ ID NO:4); TM domain (positions 165-192 of SEQ ID NO:4); and CD (positions 193-264 of SEQ ID NO:4). The proteolytic cleavage site within the SEA domain that cleaves the ED domain from the N-terminal portion of the SEA domain is between positions 106 and 107 of SEQ ID NO:4. This transcript variant does not contain the VNTR region as shown in SEQ ID NO:11.

[0074] Another nucleotide sequence encoding another human MUC1 protein is represented herein by SEQ ID NO:5, which corresponds to GENBANK® Accession No. AY327587.1 (GI:33150003). SEQ ID NO:5 encodes a 264 amino acid human MUC1 protein (transcript variant 2, also known as "MUC1/Y"), the amino acid sequence of which is represented here as SEQ ID NO:6 (also found in GENBANK® Accession No. AAP97018.1 (GI: 33150004). Within SEQ ID NO:6, the following domains are present: signal sequence (positions 1-32 of SEQ ID NO:6); SEA domain (positions 45-161 of SEQ ID NO:6); ED (positions 107 to 164 of SEQ ID NO:6); TM domain (positions 165-192 of SEQ ID NO:6); and CD (positions 193 to 264 of SEQ ID NO:6). The proteolytic cleavage site within the SEA domain that cleaves the ED domain from the N-terminal portion of the SEA domain is between positions 106 and 107 of SEQ ID NO:6. This transcript variant does not contain the VNTR region as shown in SEQ ID NO:11. SEQ ID NO:6 is 99% identical to SEQ ID NO:4, illustrating the high degree of homology among MUC1 sequences from different sources.

[0075] Another nucleotide sequence encoding another human MUC1 protein is represented herein by SEQ ID NO:7, which corresponds to GENBANK® Accession No. NM_001018017 (GI:324120954). SEQ ID NO:7 encodes a 255 amino acid human MUC1 protein (transcript variant 3), the amino acid sequence of which is represented here as SEQ ID NO:8 (also found in GENBANK® Accession No. NP_001018017.1; GI:67189069). Within SEQ ID NO:8, the following domains are present: signal sequence (positions 1-27 of SEQ ID NO:8); SEA domain (positions 36-152 of SEQ ID NO:8); ED (positions 98-155 of SEQ ID NO:8); TM domain (positions 156-183 of SEQ ID NO:8); and CD

(positions 184-255 of SEQ ID NO:8). The proteolytic cleavage site within the SEA domain that cleaves the ED domain from the N-terminal portion of the SEA domain is between positions 97 and 98 of SEQ ID NO:6. This transcript variant does not contain the VNTR region as shown in SEQ ID NO:11.

[0076] Human MUC1 has high homology with MUC1 from other animal species and therefore, one can expect to be able to identify the domains within a given MUC1 protein based on comparison of sequences. In addition, one could utilize certain sequences of MUC1 from other animal species, and particularly mammalian species, in the preparation of a Yeast-MUC1 immunotherapeutic composition of the invention, particularly where these sequences are identical or substantially homologous, and where these sequences elicit an effective immune response against the target antigen (*e.g.,* native MUC1 expressed by a tumor cell). For example, a murine MUC1 protein is represented herein by the amino acid sequence of SEQ ID NO:9. SEQ ID NO:9 corresponds to GENBANK® Accession No. NM_013605 (GI:7305292). SEQ ID NO:9 encodes a 631 amino acid murine MUC1 protein, the amino acid sequence of which is represented here as SEQ ID NO:10 (also found in GENBANK® Accession No. NP_038633; GI:7305293). Within SEQ ID NO:10, the following domains are present: signal sequence (approximately positions 1-20 of SEQ ID NO:10); VNTR (identifiable within positions 21-425 of SEQ ID NO: 10); SEA domain (positions 426-528 of SEQ ID NO: 10); ED (positions 475-536 of SEQ ID NO:10); TM domain (positions 531-559 of SEQ ID NO: 10); and CD (positions 560-631 of SEQ ID NO: 10). The proteolytic cleavage site within the SEA domain that cleaves the ED domain from the N-terminal portion of the SEA domain is between positions 474 and 475 of SEQ ID NO:10. To illustrate the level of conservation of MUC1 sequences within domains, the murine MUC1 SEA domain of SEQ ID NO:10 is 62% identical and 68% homologous or positive (as defined by BLAST) to the human MUC1 SEA domain of SEQ ID NO:11. The murine MUC1 ED of SEQ ID NO:10 is 56% identical and 73% homologous to the human MUC1 ED of SEQ ID NO:11. The murine MUC1 TM domain of SEQ ID NO:10 is 89% identical and 93% homologous to the human MUC1 TM domain of SEQ ID NO:11. The murine MUC1 CD of SEQ ID NO:10 is 88% identical and 88% homologous to the human MUC1 CD of SEQ ID NO:11.

[0077] Human MUC1, including the human MUC1 proteins and MUC1 antigens described herein, contains various $CD4^+$ and $CD8^+$ T cell epitopes. Such T cell epitopes have been described, for example, in U.S. Patent 6,546,643; U.S. Patent No. 7,118,738;

U.S. Patent No. 7,342,094; U.S. Patent No. 7,696,306; and U.S. Patent Application Publication No. 2008/0063653.

[0078] In one embodiment of the invention, a MUC1 antigen comprises or consists of a fusion protein comprising multiple domains of a MUC1 protein. In one embodiment, the MUC1 antigen is derived or designed from portions of the MUC1-C subunit. In one embodiment, the MUC1 antigen is derived or designed from portions of the MUC1-N subunit. In one embodiment, the MUC1 antigen is derived or designed from portions of both the MUC1-C and the MUC1-N subunits.

[0079] In one embodiment of the invention, a fusion protein useful in a yeast-based immunotherapeutic composition of the invention includes at least two, at least three, at least four, or at least five of the following MUC1 antigens, arranged in any order within the fusion protein, and any of which may be repeated two or more times within the fusion protein (*e.g.,* a combination of two or more CD segments): (1) a MUC1 signal sequence; (2) at least one portion of a MUC1 SEA domain and/or at least one portion of the MUC1 extracellular domain (ED) or an immunogenic domain thereof, which may include, in one aspect, most of or the entire ED in addition to flanking one or more flanking amino acids from the SEA domain; (3) at least two VNTR domains; (4) at least one MUC1 transmembrane domain or immunogenic domain thereof; and (5) at least one MUC1 cytoplasmic domain (CD) or immunogenic domain thereof. Such a fusion protein is not and does not comprise a full-length MUC1 protein (*i.e.,* it does not include a complete MUC1-N subunit and a complete MUC1-C subunit), and such a fusion protein does not comprise a full-length MUC1-N subunit. In one aspect, such a fusion protein does not comprise a full-length MUC1-C subunit. In one aspect, the segments of the fusion protein (*e.g.,* MUC1 proteins or domains, including immunogenic domains) are arranged in a different order than the arrangement of the segments as they would occur in a native or wild-type MUC1 protein.

[0080] A MUC1 signal sequence (or leader sequence) useful in a fusion protein described above or elsewhere herein can be a signal sequence from any MUC1 protein, and in one aspect, is from a human MUC1 protein. In one aspect of the invention, the MUC1 signal sequence used in a fusion protein of the invention has an amino acid sequence comprising or consisting of an amino acid sequence selected from: positions 1-27 of SEQ ID NO:2, positions 1-32 of SEQ ID NO:4, positions 1-32 of SEQ ID NO:6, positions 1-27 of SEQ ID NO:8, positions 1-23 of SEQ ID NO:11, positions 1-30 of SEQ ID NO:14, a corresponding sequence from a different MUC1 protein such as another

human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences. The MUC1 signal sequence is, in one aspect of the invention, placed at the N-terminus of a fusion protein useful in the invention. In one aspect of the invention, a non-MUC1 sequence is used in place of (instead of) a MUC1 signal sequence, such as any of the N-terminal synthetic and yeast-derived peptides described below for use with a fusion protein of the invention.

[0081] A MUC1 sea urchin sperm protein, enterokinase and agrin (SEA) domain useful in a fusion protein described above or elsewhere herein can be an SEA domain, or a portion thereof that includes at least one immunogenic domain, from any MUC1 protein, and in one aspect, is from a human MUC1 protein. In one aspect of the invention, a portion of the MUC1 SEA domain useful in a fusion protein of the invention comprises at least amino acid sequence from the extracellular domain (ED) of MUC1, but can exclude sequence upstream of the ED domain. In one aspect of the invention, the MUC1 SEA domain used in a fusion protein of the invention has an amino acid sequence comprising or consisting of an amino acid sequence selected from: positions 55-170 or positions 116-170 of SEQ ID NO:2 or at least one immunogenic domain thereof, positions 45-161 or positions 107-161 of SEQ ID NO:4 or at least one immunogenic domain thereof, positions 45-161 or positions 107-161 of SEQ ID NO:6 or at least one immunogenic domain thereof, positions 36-152 or positions 98-152 of SEQ ID NO:8 or at least one immunogenic domain thereof, positions 1034-1152 or positions 1098-1152 of SEQ ID NO:11 or at least one immunogenic domain thereof, positions 31-86 of SEQ ID NO:14 or at least one immunogenic domain thereof, positions 1-56 of SEQ ID NO:15 or at least one immunogenic domain thereof, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0082] A MUC1 extracellular domain (ED) useful in a fusion protein described above or elsewhere herein can be an ED or a portion thereof that includes at least one immunogenic domain from any MUC1 protein, and in one aspect, is from a human MUC1 protein. In one aspect of the invention, the MUC1 ED used in a fusion protein of the invention has an amino acid sequence comprising or consisting of an amino acid sequence selected from: positions 116-173 of SEQ ID NO:2 or at least one immunogenic domain thereof, positions 107-164 of SEQ ID NO:4 or at least one immunogenic domain thereof,

positions 107-164 of SEQ ID NO:6 or at least one immunogenic domain thereof, positions 98-155 of SEQ ID NO:8 or at least one immunogenic domain thereof, positions 1098-1155 of SEQ ID NO:11 or at least one immunogenic domain thereof, positions 32-89 of SEQ ID NO:14 or at least one immunogenic domain thereof, positions 2-59 of SEQ ID NO:15 or at least one immunogenic domain thereof, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0083] A MUC1 single variable number of tandem repeat (VNTR) domain useful in a fusion protein described above or elsewhere herein can be a VNTR domain from any MUC1 protein, and in one aspect, is from a human MUC1 protein. In one aspect of the invention, the MUC1 VNTR domain used in a fusion protein of the invention has an amino acid sequence comprising or consisting of an amino acid sequence selected from: positions 126-145 of SEQ ID NO:11, any consecutive 20 amino acids between positions 61 and 1020 of SEQ ID NO:11 or any consecutive 20 amino acids between positions 126 and 965 of SEQ ID NO:11; SEQ ID NO:12 (including any of the polymorphisms within SEQ ID NO:12 as described above), any consecutive 20 amino acids between positions 90 and 130 of SEQ ID NO:14, any consecutive 20 amino acids between positions 60 and 100 of SEQ ID NO:15, a corresponding VNTR sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0084] A MUC1 transmembrane (TM) domain useful in a fusion protein described above or elsewhere herein can be a TM domain or a portion thereof that includes at least one immunogenic domain from any MUC1 protein, and in one aspect, is from a human MUC1 protein. In one aspect of the invention, the MUC1 TM domain used in a fusion protein of the invention has an amino acid sequence comprising or consisting of an amino acid sequence selected from: positions 174-201 of SEQ ID NO:2 or at least one immunogenic domain thereof, positions 165-192 of SEQ ID NO:4 or at least one immunogenic domain thereof, positions 165-192 of SEQ ID NO:6 or at least one immunogenic domain thereof, positions 156-183 of SEQ ID NO:8 or at least one immunogenic domain thereof, positions 1156-1183 of SEQ ID NO:11 or at least one immunogenic domain thereof, positions 131-158 of SEQ ID NO:14 or at least one immunogenic domain thereof, positions 101-128 of SEQ ID NO:15 or at least one

immunogenic domain thereof, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0085] A MUC1 cytoplasmic domain (CD) useful in a fusion protein described above or elsewhere herein can be a CD or a portion thereof that includes at least one immunogenic domain from any MUC1 protein, and in one aspect, is from a human MUC1 protein. In one aspect of the invention, the MUC1 CD used in a fusion protein of the invention has an amino acid sequence comprising or consisting of an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2 or at least one immunogenic domain thereof, positions 193-264 of SEQ ID NO:4 or at least one immunogenic domain thereof, positions 193-264 of SEQ ID NO:6 or at least one immunogenic domain thereof, positions 184-255 of SEQ ID NO:8 or at least one immunogenic domain thereof, positions 1184-1255 of SEQ ID NO:11 or at least one immunogenic domain thereof, positions 159-230 of SEQ ID NO:14 or at least one immunogenic domain thereof, positions 129-200 of SEQ ID NO:15 or at least one immunogenic domain thereof, positions 7-78 or positions 79-150 or positions 151-222 of SEQ ID NO:17 or at least one immunogenic domain thereof; positions 1-72 or positions 73-144 or positions 145-216 of SEQ ID NO:18 or an immunogenic domain thereof, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0086] In one embodiment of the invention, a fusion protein useful in a yeast-based immunotherapeutic composition of the invention includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a portion of a MUC1 SEA domain and MUC1 extracellular domain (ED) that includes most or all of the MUC1 ED; (2) at least two VNTR domains; (3) a MUC1 transmembrane domain; and (4) a MUC1 cytoplasmic domain (CD). In one embodiment, the fusion protein includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a MUC1 signal sequence; (2) a portion of a MUC1 SEA domain and MUC1 extracellular domain (ED) that includes most or all of the MUC1 ED; (3) at least two VNTR domains; (4) a MUC1 transmembrane domain; and (5) a MUC1 cytoplasmic domain (CD). Additional or alternate elements to be included in a fusion protein of the invention may include N-terminal and/or C-terminal peptides that improve or assist with the expression or stability of, and/or allow for

identification and/or purification of, the fusion protein, and/or short intervening linker sequences (*e.g.,* 1, 2, 3, 4, or 5 amino acid peptides) between segments of the fusion protein which can be useful for the introduction of restriction enzyme sites to facilitate cloning, as cleavage sites for host phagosomal proteases, to accelerate protein or antigen processing, and for future manipulation of the constructs. Such elements are described in detail below.

[0087] One example of such a fusion protein that is useful in a yeast-based immunotherapeutic composition of the invention comprises or includes the following MUC1 antigens, in the following order from N- to C-terminus:

(1) a MUC1 extracellular domain (ED) that may be appended at the N-terminus by one, two, three, four, five or more flanking amino acids from the non-ED portion of the SEA domain that reside upstream of the ED in the wild-type protein, wherein the ED segment comprises or consists of an amino acid sequence selected from: positions 116-173 of SEQ ID NO:2; or positions 107-164 of SEQ ID NO:4; positions 107-164 of SEQ ID NO:6; positions 98-155 of SEQ ID NO:8; positions 1098-1155 of SEQ ID NO:11; positions 32-89 of SEQ ID NO:14; positions 2-59 of SEQ ID NO:15; a corresponding sequence from a different MUC1 protein such as another human MUC1 protein; or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences;

(2) at least two VNTR domains, wherein each of the VNTR domains comprise or consist of an amino acid sequence selected from: positions 126-145 of SEQ ID NO:11, any consecutive 20 amino acids between positions 61 and 1020 of SEQ ID NO:11 or any consecutive 20 amino acids between positions 126 and 965 of SEQ ID NO:11; SEQ ID NO:12 (including any of the polymorphisms within SEQ ID NO:12 as described above), any consecutive 20 amino acids between positions 90 and 130 of SEQ ID NO:14, any consecutive 20 amino acids between positions 60 and 100 of SEQ ID NO:15, a corresponding VNTR sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences;

(3) a MUC1 transmembrane (TM) domain, wherein the TM domain comprises or consists of an amino acid sequence selected from: positions 174-201 of SEQ ID

NO:2 , positions 165-192 of SEQ ID NO:4, positions 165-192 of SEQ ID NO:6, positions 156-183 of SEQ ID NO:8, positions 1156-1183 of SEQ ID NO:11, positions 131-158 of SEQ ID NO:14, positions 101-128 of SEQ ID NO:15, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences; and

(4) a MUC1 cytoplasmic domain (CD), wherein the CD comprises or consists of an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2, positions 193-264 of SEQ ID NO:4, positions 193-264 of SEQ ID NO:6, positions 184-255 of SEQ ID NO:8, positions 1184-1255 of SEQ ID NO:11, positions 159-230 of SEQ ID NO:14, positions 129-200 of SEQ ID NO:15, positions 7-78 or positions 79-150 or positions 151-222 of SEQ ID NO:17; positions 1-72 or positions 73-144 or positions 145-216 of SEQ ID NO:18, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0088] In one aspect of this embodiment, a fusion protein that is useful in a yeast-based immunotherapeutic composition of the invention comprises or consists of the amino acid sequence of SEQ ID NO:14 or SEQ ID NO:15. SEQ ID NO:14 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a MUC1 signal sequence (positions 1-30 of SEQ ID NO:14); (2) a MUC1 SEA/ED segment (positions 31-89 of SEQ ID NO:14); (3) a VNTR segment comprising two VNTR domains (positions 90-130 of SEQ ID NO:14); (4) a MUC1 TM domain (positions 131-158 of SEQ ID NO:14); (5) a MUC1 CD (positions 159-230 of SEQ ID NO:14); and (6) a hexapeptide histidine tag (positions 231-236 of SEQ ID NO:14). SEQ ID NO:14 is encoded by the nucleotide sequence represented by SEQ ID NO:13. SEQ ID NO:15 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a MUC1 SEA/ED segment (positions 1-59 of SEQ ID NO:15); (2) a VNTR segment comprising two VNTR domains (positions 60-100 of SEQ ID NO:15); (3) a MUC1 TM domain (positions 101-128 of SEQ ID NO:15); and (4) a MUC1 CD (positions 129-200 of SEQ ID NO:15). Optionally, the fusion protein of SEQ ID NO:14 or SEQ ID NO:15 includes an N-terminal peptide that is a synthetic N-terminal peptide designed to impart

resistance to proteasomal degradation and stabilize expression represented by SEQ ID NO:21, or an N-terminal peptide from a yeast alpha leader sequence such as SEQ ID NO:19 or SEQ ID NO:20, or another N-terminal peptide suitable for use with a yeast-based immunotherapeutic as described herein. Also optionally, one or more linker peptides of from one, two, three or more amino acids, such as the two amino acid linker of Thr-Ser, can be inserted between segments of the fusion protein. The hexahistidine tag at the C-terminus of the fusion protein is also optional.

[0089] In one embodiment of the invention, a fusion protein useful in a yeast-based immunotherapeutic composition of the invention includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) at least two VNTR domains; and (2) a portion of a MUC1 SEA domain and MUC1 extracellular domain (ED) that includes most or all of the MUC1 ED. Such a fusion protein may include additional portions of the MUC1-N region flanking the VNTR domains. Such a fusion protein does not include an entire MUC1-N subunit.

[0090] In another embodiment of the invention, a fusion protein useful in a yeast-based immunotherapeutic composition of the invention includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a first MUC1 cytoplasmic domain (CD); (2) a second MUC1 cytoplasmic domain (CD); and (3) a third MUC1 cytoplasmic domain (CD). In one embodiment, additional MUC1 cytoplasmic domains can be included in such a fusion protein. In one embodiment, at least one of the MUC1 CDs is from a different source than one of the other MUC1 CDs (*e.g.,* one MUC1 CD is from a first human MUC1 protein and one MUC1 CD is from a second MUC1 protein, wherein there can be sequence differences between the first and second MUC1 CDs). In one embodiment, this fusion protein is appended at the N-terminus with a MUC1 signal sequence. In another embodiment, this fusion protein may include N-terminal and/or C-terminal peptides that improve or assist with the expression or stability of, and/or allow for identification and/or purification of, the fusion protein, and/or short intervening linker sequences (*e.g.,* 1, 2, 3, 4, or 5 amino acid peptides) between segments of the fusion protein which can be useful for the introduction of restriction enzyme sites to facilitate cloning, as cleavage sites for host phagosomal proteases, to accelerate protein or antigen processing, and for future manipulation of the constructs.

[0091] One example of such a fusion protein that is useful in a yeast-based immunotherapeutic composition of the invention comprises or includes the following MUC1 antigens, in the following order from N- to C-terminus:

(1) a first MUC1 cytoplasmic domain (CD), wherein the CD comprises or consists of an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2, positions 193-264 of SEQ ID NO:4, positions 193-264 of SEQ ID NO:6, positions 184-255 of SEQ ID NO:8, positions 1184-1255 of SEQ ID NO:11, positions 159-230 of SEQ ID NO:14, positions 129-200 of SEQ ID NO:15, positions 7-78 or positions 79-150 or positions 151-222 of SEQ ID NO:17; positions 1-72 or positions 73-144 or positions 145-216 of SEQ ID NO:18, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences;

(2) a second MUC1 cytoplasmic domain (CD), wherein the CD comprises or consists of an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2, positions 193-264 of SEQ ID NO:4, positions 193-264 of SEQ ID NO:6, positions 184-255 of SEQ ID NO:8, positions 1184-1255 of SEQ ID NO:11, positions 159-230 of SEQ ID NO:14, positions 129-200 of SEQ ID NO:15, positions 7-78 or positions 79-150 or positions 151-222 of SEQ ID NO:17; positions 1-72 or positions 73-144 or positions 145-216 of SEQ ID NO:18, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences;

(3) a third MUC1 cytoplasmic domain (CD), wherein the CD comprises or consists of an amino acid sequence selected from: positions 202-273 of SEQ ID NO:2, positions 193-264 of SEQ ID NO:4, positions 193-264 of SEQ ID NO:6, positions 184-255 of SEQ ID NO:8, positions 1184-1255 of SEQ ID NO:11, positions 159-230 of SEQ ID NO:14, positions 129-200 of SEQ ID NO:15, positions 7-78 or positions 79-150 or positions 151-222 of SEQ ID NO:17; positions 1-72 or positions 73-144 or positions 145-216 of SEQ ID NO:18, a corresponding sequence from a different MUC1 protein such as another human MUC1 protein, or an amino acid sequence that is at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical, to any one of these amino acid sequences.

[0092] In one aspect of this embodiment, a fusion protein that is useful in a yeast-based immunotherapeutic composition of the invention comprises or consists of the amino acid sequence of SEQ ID NO:17 or SEQ ID NO:18. SEQ ID NO:17 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) an synthetic peptide represented by SEQ ID NO:21 (positions 1-6 of SEQ ID NO:17); (2) a first MUC1 CD (positions 7-78 of SEQ ID NO:17); (3) a second MUC1 CD (positions 79-150 of SEQ ID NO:17); (4) a third MUC1 CD (positions 151-222 of SEQ ID NO:17); and (5) a hexahistidine tag (positions 223-228 of SEQ ID NO:17). SEQ ID NO:17 is encoded by the nucleotide sequence represented by SEQ ID NO:16. SEQ ID NO:18 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a first MUC1 CD (positions 1-72 of SEQ ID NO:18); (2) a second MUC1 CD (positions 73-144 of SEQ ID NO:18); (3) a third MUC1 CD (positions 145-216 of SEQ ID NO:18). Optionally, the fusion protein of SEQ ID NO:17 or SEQ ID NO:18 includes an N-terminal peptide that is a synthetic N-terminal peptide designed to impart resistance to proteasomal degradation and stabilize expression represented by SEQ ID NO:21, or an N-terminal peptide from a yeast alpha leader sequence such as SEQ ID NO:19 or SEQ ID NO:20, or another N-terminal peptide suitable for use with a yeast-based immunotherapeutic as described herein. Also optionally, one or more linker peptides of from one to three or more amino acids linker sequences of one, two, three or more amino acids, such as the two amino acid linker of Thr-Ser can be inserted between segments of the fusion protein. The hexahistidine tag at the C-terminus of the fusion protein is also optional.

[0093] A MUC1 antigen useful in the present invention also includes proteins having an amino acid sequence that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of any of the MUC1 proteins, domains, fusion proteins, or antigens described herein over the full length of the protein, or with respect to a defined fragment or domain thereof (*e.g.,* an immunological domain or functional domain (domain with at least one biological activity)) that forms part of the protein. For example, a domain of the MUC1 protein described herein includes the signal sequence, a VNTR domain, an SEA domain, the extracellular domain (ED), an TM domain, and/or a cytoplasmic domain (CD). An immunological domain has been described in detail above. MUC1 fusion proteins described herein include those represented by SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:17 and SEQ ID NO:18. Accordingly, a MUC1 antigen useful in the yeast-based immunotherapeutic of the invention includes a MUC1 antigen comprising or consisting of

any one of SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:17 and SEQ ID NO:18, an amino acid sequence that is at least about 95%, 96%, 97%, 98% or 99% identical to any one of SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:17 and SEQ ID NO:18, and/or a corresponding sequence from a different MUC1 protein (*e.g.,* a fusion protein where one or more of the fusion segments are from the corresponding sequences from a different MUC1 protein or from a MUC1 protein agonist sequence, such that minor sequence differences may be present as compared to the sequences presented here).

[0094] It is straightforward to use the corresponding portions of any of the MUC1 proteins or domains derived or obtained from sequence or sources other than those exemplified herein, and particularly from sequences or sources within the same animal species, to create fusion proteins having a similar or the same overall structure as the fusion proteins described herein. By way of example, one can readily identify a corresponding sequence in given human MUC1 protein from any source that corresponds to positions 1034-1152 of SEQ ID NO:11, using simple sequence alignment tools or processes. Therefore, sequences with minor and/or conservative differences from the sequences exemplified herein are expressly encompassed by the present invention.

[0095] In some aspects of the invention, amino acid insertions, deletions, and/or substitutions can be made for one, two, three, four, five, six, seven, eight, nine, ten, eleven, or more amino acids of a wild-type or reference MUC1 protein, provided that the resulting MUC1 protein, when used as an antigen in a Yeast-MUC1 immunotherapeutic composition of the invention, elicits an immune response against a native MUC1 protein as the wild-type or reference MUC1 protein, which may include an enhanced immune response, a diminished immune response, or a substantially similar immune response. For example, the invention includes the use of MUC1 agonist antigens, which may include one or more T cell epitopes that have been mutated to enhance the T cell response against the MUC1 agonist, such as by improving the avidity or affinity of the epitope for an MHC molecule or for the T cell receptor that recognizes the epitope in the context of MHC presentation. MUC1 agonists may therefore improve the potency or efficiency of a T cell response against native MUC1 expressed by a tumor cell. A variety of MUC1 T cell epitopes, including agonist epitopes are described in U.S. Patent 6,546,643; U.S. Patent No. 7,118,738; U.S. Patent No. 7,342,094; U.S. Patent No. 7,696,306; and U.S. Patent Application Publication No. 2008/0063653, and any one or more of these epitopes can be used in a MUC1 antigen of the present invention, including by adding, deleting or

substituting one or more amino acids within a sequence described herein to conform the sequence to the published epitope sequence at that position(s).

[0096] Examples of MUC1 agonist antigens are provided herein (see Examples 3 and 4). In one embodiment, a MUC1 agonist antigen suitable for use in the present invention comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or all 11 of the following substitutions, where the positions of the substitutions are provided with respect to a wild-type MUC1 represented by Accession No. NP_001191214 (although the same or equivalent positions can be readily identified in any other wild-type MUC1 sequence): T93, A161, P162, G169, S170, T171, A392, C406, T444, D445, or S460. In one embodiment, a MUC1 agonist antigen that is useful in a yeast-based immunotherapeutic composition of the invention comprises or consists of the amino acid sequence of SEQ ID NO:23 or SEQ ID NO:25. SEQ ID NO:23 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) MUC1 signal sequence (positions 1-30 of SEQ ID NO:23); (2) a MUC1 SEA/ED segment (positions 31-89 of SEQ ID NO:23); (3) a VNTR segment comprising two VNTR domains (positions 90-130 of SEQ ID NO:23); (4) a MUC1 TM domain (positions 131-158 of SEQ ID NO:23); (5) a MUC1 CD (positions 159-230 of SEQ ID NO:23); (6) a MUC1 agonist epitope (positions 231-246 of SEQ ID NO:23) and (7) a hexapeptide histidine tag (positions 247-252 of SEQ ID NO:23). SEQ ID NO:23 is encoded by the nucleotide sequence represented by SEQ ID NO:22 (codon optimized for yeast expression). The MUC1 signal sequence (positions 1-30 of SEQ ID NO:23) could be substituted with a different N-terminal sequence designed to impart resistance to proteasomal degradation and/or stabilize expression, such as the peptide represented by SEQ ID NO:21, or an N-terminal peptide from a yeast alpha leader sequence such as SEQ ID NO:19 or SEQ ID NO:20. hexahistidine C-terminal tag is optional, and facilitates identification and/or purification of the protein. As compared to the MUC1 antigen in SEQ ID NO:14 or SEQ ID NO:15, the SEQ ID NO:23 contains the following amino acid substitutions to create a variety of agonist epitopes (substitution positions given with reference to SEQ ID NO:23, with further reference to the location of the substitution in a wild-type MUC1 represented by Accession No. NP_001191214): A96Y (position 161 in wild-type MUC1), P97L (position 162 in wild-type MUC1), G104V (position 169 in wild-type MUC1), S105Y (position 170 in wild-type MUC1), T106L (position 171 in wild-type MUC1), A147Y (position 392 in wild-type MUC1), C161V (position 406 in wild-type MUC1), T199L (position 444 in wild-type MUC1), D200F (position 445 in wild-type

MUC1), S215Y (position 460 in wild-type MUC1), and T239L (position 93 in wild-type MUC1).

[0097] SEQ ID NO:25 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) an alpha factor leader sequence disclosed elsewhere herein by SEQ ID NO:19 (positions 1-89 of SEQ ID NO:25); (2) a linker sequence of Thr-Ser (positions 90-91 of SEQ ID NO:25); (3) a full-length MUC1 agonist protein corresponding to a wild-type protein except for the introduction of 11 agonist epitopes (positions 92-566 of SEQ ID NO:25) and (7) a hexapeptide histidine tag (positions 567-572 of SEQ ID NO:25). SEQ ID NO:25 is encoded by the nucleotide sequence represented by SEQ ID NO:24 (codon optimized for yeast expression). The alpha leader sequence (positions 1-89 of SEQ ID NO:25) could be substituted with a different N-terminal sequence designed to impart resistance to proteasomal degradation and/or stabilize expression, such as the peptide represented by SEQ ID NO:21, or an N-terminal peptide from a different yeast alpha leader sequence such as SEQ ID NO:20, or by a MUC1 signal sequence. The hexahistidine C-terminal tag is optional, and facilitates identification and/or purification of the protein. As compared to the wild-type MUC1 protein used as a template, the sequence in GI-6106 contains the following amino acid substitutions to create a variety of agonist epitopes (substitution positions given with reference to SEQ ID NO:25, with further reference to the location of the substitution in a wild-type MUC1 represented by Accession No. NP_001191214): T184L (position 93 in wild-type MUC1), A232Y (position 161 in wild-type MUC1), P233L (position 162 in wild-type MUC1), G240V (position 169 in wild-type MUC1), S241Y (position 170 in wild-type MUC1), T242L (position 171 in wild-type MUC1), A483Y (position 392 in wild-type MUC1), C497V (position 406 in wild-type MUC1), T535L (position 444 in wild-type MUC1), D536F (position 445 in wild-type MUC1), and S551Y (position 460 in wild-type MUC1).

[0098] Accordingly, a MUC1 agonist antigen useful in the yeast-based immunotherapeutic of the invention includes a MUC1 antigen comprising or consisting of SEQ ID NO:23 or SEQ ID NO:25, or the MUC1-specific sequences within these larger fusion proteins, or an amino acid sequence that is at least about 95%, 96%, 97%, 98% or 99% identical to any one of SEQ ID NO:23, SEQ ID NO:25, or the MUC1-specific sequences within these larger fusion proteins, and/or a corresponding sequence from a different MUC1 protein (*e.g.,* a fusion protein where one or more of the fusion segments

are from the corresponding sequences from a different MUC1 protein, such that minor sequence differences may be present as compared to the sequences presented here).

[0099] As discussed above, N-terminal expression sequences and the C-terminal tags, such as those described above with respect to the fusion proteins of SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:23, or SEQ ID NO:25 are optional, but may be selected from several different sequences described elsewhere herein to improve or assist with expression, stability, and/or allow for identification and/or purification of the protein. Also, many different promoters suitable for use in yeast are known in the art. Furthermore, short intervening linker sequences (*e.g.,* 1, 2, 3, 4, or 5 amino acid peptides) may be introduced between portions of a fusion protein comprising a MUC1 antigen for a variety of reasons, including the introduction of restriction enzyme sites to facilitate cloning, as cleavage sites for host phagosomal proteases, to accelerate protein or antigen processing, and for future manipulation of the constructs.

[00100] Optionally, proteins, including fusion proteins, which are used as a component of the Yeast-MUC1 immunotherapeutic composition of the invention are produced using antigen constructs that are particularly useful for improving or stabilizing the expression of heterologous antigens in yeast. In one embodiment, the desired antigenic protein(s) or peptide(s) are fused at their amino-terminal end to: (a) a specific synthetic peptide that stabilizes the expression of the fusion protein in the yeast vehicle or prevents posttranslational modification of the expressed fusion protein (such peptides are described in detail, for example, in U.S. Patent Publication No. 2004-0156858 A1, published August 12, 2004, incorporated herein by reference in its entirety); (b) at least a portion of an endogenous yeast protein, wherein either fusion partner provides improved stability of expression of the protein in the yeast and/or a prevents post-translational modification of the proteins by the yeast cells (such proteins are also described in detail, for example, in U.S. Patent Publication No. 2004-0156858 A1, supra); and/or (c) at least a portion of a yeast protein that causes the fusion protein to be expressed on the surface of the yeast (*e.g.,* an Aga protein, described in more detail herein). In addition, the present invention optionally includes the use of peptides that are fused to the C-terminus of the antigen-encoding construct, particularly for use in the selection and identification of the protein. Such peptides include, but are not limited to, any synthetic or natural peptide, such as a peptide tag (*e.g.,* 6X His or hexapeptide) or any other short epitope tag. Peptides attached to the C-terminus of an antigen according to the invention can be used with or without the addition of the N-terminal peptides discussed above, and vice versa.

[00101] In one embodiment, a synthetic peptide useful in a fusion protein to be expressed in a yeast is linked to the N-terminus of the antigen, the peptide consisting of at least two amino acid positions that are heterologous to the antigen, wherein the peptide stabilizes the expression of the fusion protein in the yeast vehicle or prevents posttranslational modification of the expressed fusion protein. The synthetic peptide and N-terminal portion of the antigen together form a fusion protein that has the following requirements: (1) the amino acid residue at position one of the fusion protein is a methionine (*i.e.*, the first amino acid in the synthetic peptide is a methionine); (2) the amino acid residue at position two of the fusion protein is not a glycine or a proline (*i.e.,* the second amino acid in the synthetic peptide is not a glycine or a proline); (3) none of the amino acid positions at positions 2-6 of the fusion protein is a methionine (*i.e.,* the amino acids at positions 2-6, whether part of the synthetic peptide or the protein, if the synthetic peptide is shorter than 6 amino acids, do not include a methionine); and (4) none of the amino acids at positions 2-6 of the fusion protein is a lysine or an arginine (*i.e.,* the amino acids at positions 2-6, whether part of the synthetic peptide or the protein, if the synthetic peptide is shorter than 5 amino acids, do not include a lysine or an arginine). The synthetic peptide can be as short as two amino acids, but in one aspect, is 2-6 amino acids (including 3, 4, 5 amino acids), and can be longer than 6 amino acids, in whole integers, up to about 200 amino acids, 300 amino acids, 400 amino acids, 500 amino acids, or more.

[00102] In one embodiment, a fusion protein comprises an amino acid sequence of M-X2-X3-X4-X5-X6, wherein M is methionine; wherein X2 is any amino acid except glycine, proline, lysine or arginine; wherein X3 is any amino acid except methionine, lysine or arginine; wherein X4 is any amino acid except methionine, lysine or arginine; wherein X5 is any amino acid except methionine, lysine or arginine; and wherein X6 is any amino acid except methionine, lysine or arginine. In one embodiment, the X6 residue is a proline. An exemplary synthetic sequence that enhances the stability of expression of an antigen in a yeast cell and/or prevents post-translational modification of the protein in the yeast includes the sequence M-A-D-E-A-P (represented herein by SEQ ID NO:21). In addition to the enhanced stability of the expression product, this fusion partner does not appear to negatively impact the immune response against the immunizing antigen in the construct. In addition, the synthetic fusion peptides can be designed to provide an epitope that can be recognized by a selection agent, such as an antibody.

[00103] In one embodiment, the MUC1 antigen is linked at the N-terminus to a yeast protein, such as an alpha factor prepro sequence (also referred to as the alpha factor signal leader sequence, the amino acid sequence of which is exemplified herein by SEQ ID NO:19 or SEQ ID NO:20 Other sequences for yeast alpha factor prepro sequence are known in the art and are encompassed for use in the present invention.

[00104] In one aspect of the invention, the yeast vehicle is manipulated such that the antigen is expressed or provided by delivery or translocation of an expressed protein product, partially or wholly, on the surface of the yeast vehicle (extracellular expression). One method for accomplishing this aspect of the invention is to use a spacer arm for positioning one or more protein(s) on the surface of the yeast vehicle. For example, one can use a spacer arm to create a fusion protein of the antigen(s) or other protein of interest with a protein that targets the antigen(s) or other protein of interest to the yeast cell wall. For example, one such protein that can be used to target other proteins is a yeast protein (*e.g.*, cell wall protein 2 (cwp2), Aga2, Pir4 or Flo1 protein) that enables the antigen(s) or other protein to be targeted to the yeast cell wall such that the antigen or other protein is located on the surface of the yeast. Proteins other than yeast proteins may be used for the spacer arm; however, for any spacer arm protein, it is most desirable to have the immunogenic response be directed against the target antigen rather than the spacer arm protein. As such, if other proteins are used for the spacer arm, then the spacer arm protein that is used should not generate such a large immune response to the spacer arm protein itself such that the immune response to the target antigen(s) is overwhelmed. One of skill in the art should aim for a small immune response to the spacer arm protein relative to the immune response for the target antigen(s). Spacer arms can be constructed to have cleavage sites (*e.g.*, protease cleavage sites) that allow the antigen to be readily removed or processed away from the yeast, if desired. Any known method of determining the magnitude of immune responses can be used (*e.g.,* antibody production, lytic assays, etc.) and are readily known to one of skill in the art.

[00105] Another method for positioning the target antigen(s) or other proteins to be exposed on the yeast surface is to use signal sequences such as glycosylphosphatidyl inositol (GPI) to anchor the target to the yeast cell wall. Alternatively, positioning can be accomplished by appending signal sequences that target the antigen(s) or other proteins of interest into the secretory pathway via translocation into the endoplasmic reticulum (ER) such that the antigen binds to a protein which is bound to the cell wall (*e.g.*, cwp).

[00106] In one aspect, the spacer arm protein is a yeast protein. The yeast protein can consist of between about two and about 800 amino acids of a yeast protein. In one embodiment, the yeast protein is about 10 to 700 amino acids. In another embodiment, the yeast protein is about 40 to 600 amino acids. Other embodiments of the invention include the yeast protein being at least 250 amino acids, at least 300 amino acids, at least 350 amino acids, at least 400 amino acids, at least 450 amino acids, at least 500 amino acids, at least 550 amino acids, at least 600 amino acids, or at least 650 amino acids. In one embodiment, the yeast protein is at least 450 amino acids in length. Another consideration for optimizing antigen surface expression, if that is desired, is whether the antigen and spacer arm combination should be expressed as a monomer or as dimer or as a trimer, or even more units connected together. This use of monomers, dimers, trimers, etc. allows for appropriate spacing or folding of the antigen such that some part, if not all, of the antigen is displayed on the surface of the yeast vehicle in a manner that makes it more immunogenic.

[00107] Use of yeast proteins can stabilize the expression of fusion proteins in the yeast vehicle, prevents posttranslational modification of the expressed fusion protein, and/or targets the fusion protein to a particular compartment in the yeast (*e.g.,* to be expressed on the yeast cell surface). For delivery into the yeast secretory pathway, exemplary yeast proteins to use include, but are not limited to: Aga (including, but not limited to, Agal and/or Aga2); SUC2 (yeast invertase); alpha factor signal leader sequence; CPY; Cwp2p for its localization and retention in the cell wall; BUD genes for localization at the yeast cell bud during the initial phase of daughter cell formation; Flo1p; Pir2p; and Pir4p.

[00108] Other sequences can be used to target, retain and/or stabilize the protein to other parts of the yeast vehicle, for example, in the cytosol or the mitochondria or the endoplasmic reticulum or the nucleus. Examples of suitable yeast protein that can be used for any of the embodiments above include, but are not limited to, TK, AF, SEC7; phosphoenolpyruvate carboxykinase PCK1, phosphoglycerokinase PGK and triose phosphate isomerase TPI gene products for their repressible expression in glucose and cytosolic localization; the heat shock proteins SSA1, SSA3, SSA4, SSC1, whose expression is induced and whose proteins are more thermostable upon exposure of cells to heat treatment; the mitochondrial protein CYC1 for import into mitochondria; ACT1.

[00109] Methods of producing yeast vehicles and expressing, combining and/or associating yeast vehicles with antigens and/or other proteins and/or agents of interest to produce yeast-based immunotherapy compositions are contemplated by the invention.

[00110] According to the present invention, the term "yeast vehicle-antigen complex" or "yeast-antigen complex" is used generically to describe any association of a yeast vehicle with an antigen, and can be used interchangeably with "yeast-based immunotherapy composition" when such composition is used to elicit an immune response as described above. Such association includes expression of the antigen by the yeast (a recombinant yeast), introduction of an antigen into a yeast, physical attachment of the antigen to the yeast, and mixing of the yeast and antigen together, such as in a buffer or other solution or formulation. These types of complexes are described in detail below.

[00111] In one embodiment, a yeast cell used to prepare the yeast vehicle is transfected with a heterologous nucleic acid molecule encoding a protein (*e.g.,* the antigen) such that the protein is expressed by the yeast cell. Such a yeast is also referred to herein as a recombinant yeast or a recombinant yeast vehicle. The yeast cell can then be formulated with a pharmaceutically acceptable excipient and administered directly to a patient, stored for later administration, or loaded into a dendritic cell as an intact cell. The yeast cell can also be killed, or it can be derivatized such as by formation of yeast spheroplasts, cytoplasts, ghosts, or subcellular particles, any of which may be followed by storing, administering, or loading of the derivative into the dendritic cell. Yeast spheroplasts can also be directly transfected with a recombinant nucleic acid molecule (*e.g.*, the spheroplast is produced from a whole yeast, and then transfected) in order to produce a recombinant spheroplast that expresses the antigen. Yeast cells or yeast spheroplasts that recombinantly express the antigen(s) may be used to produce a yeast vehicle comprising a yeast cytoplast, a yeast ghost, or a yeast membrane particle or yeast cell wall particle, or fraction thereof.

[00112] In general, the yeast vehicle and antigen(s) and/or other agents can be associated by any technique described herein. In one aspect, the yeast vehicle was loaded intracellularly with the antigen(s) and/or agent(s). In another aspect, the antigen(s) and/or agent(s) was covalently or non-covalently attached to the yeast vehicle. In yet another aspect, the yeast vehicle and the antigen(s) and/or agent(s) were associated by mixing. In another aspect, and in one embodiment, the antigen(s) and/or agent(s) are expressed recombinantly by the yeast vehicle or by the yeast cell or yeast spheroplast from which the yeast vehicle was derived.

[00113] A number of antigens and/or other proteins to be produced by a yeast vehicle of the present invention is any number of antigens and/or other proteins that can be reasonably produced by a yeast vehicle, and typically ranges from at least one to at least about 6 or more, including from about 2 to about 6 antigens and or other proteins.

[00114] Expression of an antigen or other protein in a yeast vehicle of the present invention is accomplished using techniques known to those skilled in the art. Briefly, a nucleic acid molecule encoding at least one desired antigen or other protein is inserted into an expression vector in such a manner that the nucleic acid molecule is operatively linked to a transcription control sequence in order to be capable of effecting either constitutive or regulated expression of the nucleic acid molecule when transformed into a host yeast cell. Nucleic acid molecules encoding one or more antigens and/or other proteins can be on one or more expression vectors operatively linked to one or more expression control sequences. Particularly important expression control sequences are those which control transcription initiation, such as promoter and upstream activation sequences. Any suitable yeast promoter can be used in the present invention and a variety of such promoters are known to those skilled in the art. Promoters for expression in *Saccharomyces cerevisiae* include, but are not limited to, promoters of genes encoding the following yeast proteins: alcohol dehydrogenase I (ADH1) or II (ADH2), CUP1, phosphoglycerate kinase (PGK), triose phosphate isomerase (TPI), translational elongation factor EF-1 alpha (TEF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; also referred to as TDH3, for triose phosphate dehydrogenase), galactokinase (GAL1), galactose-1-phosphate uridyl-transferase (GAL7), UDP-galactose epimerase (GAL10), cytochrome c1 (CYC1), Sec7 protein (SEC7) and acid phosphatase (PHO5), including hybrid promoters such as *ADH2/GAPDH* and *CYC1/GAL10* promoters, and including the *ADH2/GAPDH* promoter, which is induced when glucose concentrations in the cell are low (*e.g.,* about 0.1 to about 0.2 percent), as well as the *CUP1* promoter and the *TEF2* promoter. Likewise, a number of upstream activation sequences (UASs), also referred to as enhancers, are known. Upstream activation sequences for expression in *Saccharomyces cerevisiae* include, but are not limited to, the UASs of genes encoding the following proteins: PCK1, TPI, TDH3, CYC1, ADH1, ADH2, SUC2, GAL1, GAL7 and GAL10, as well as other UASs activated by the GAL4 gene product, with the ADH2 UAS being used in one aspect. Since the ADH2 UAS is activated by the ADR1 gene product, it may be preferable to overexpress the ADR1 gene when a heterologous gene is operatively linked to the ADH2 UAS.

Transcription termination sequences for expression in *Saccharomyces cerevisiae* include the termination sequences of the α-factor, GAPDH, and CYC1 genes.

[00115] Transcription control sequences to express genes in methyltrophic yeast include the transcription control regions of the genes encoding alcohol oxidase and formate dehydrogenase.

[00116] Transfection of a nucleic acid molecule into a yeast cell according to the present invention can be accomplished by any method by which a nucleic acid molecule can be introduced into the cell and includes, but is not limited to, diffusion, active transport, bath sonication, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. Transfected nucleic acid molecules can be integrated into a yeast chromosome or maintained on extrachromosomal vectors using techniques known to those skilled in the art. Examples of yeast vehicles carrying such nucleic acid molecules are disclosed in detail herein. As discussed above, yeast cytoplast, yeast ghost, and yeast membrane particles or cell wall preparations can also be produced recombinantly by transfecting intact yeast microorganisms or yeast spheroplasts with desired nucleic acid molecules, producing the antigen therein, and then further manipulating the microorganisms or spheroplasts using techniques known to those skilled in the art to produce cytoplast, ghost or subcellular yeast membrane extract or fractions thereof containing desired antigens or other proteins.

[00117] Effective conditions for the production of recombinant yeast vehicles and expression of the antigen and/or other protein by the yeast vehicle include an effective medium in which a yeast strain can be cultured. An effective medium is typically an aqueous medium comprising assimilable carbohydrate, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals and other nutrients, such as vitamins and growth factors. The medium may comprise complex nutrients or may be a defined minimal medium. Yeast strains of the present invention can be cultured in a variety of containers, including, but not limited to, bioreactors, Erlenmeyer flasks, test tubes, microtiter dishes, and Petri plates. Culturing is carried out at a temperature, pH and oxygen content appropriate for the yeast strain. Such culturing conditions are well within the expertise of one of ordinary skill in the art (see, for example, Guthrie et al. (eds.), 1991, Methods in Enzymology, vol. 194, Academic Press, San Diego). For example, under one protocol, liquid cultures containing a suitable medium can be inoculated using cultures obtained from starter plates and/or starter cultures of Yeast-MUC1 immunotherapy compositions, and are grown for approximately 20h at 30°C, with agitation at 250 rpm. Primary cultures

can then be expanded into larger cultures as desired. Protein expression from vectors with which the yeast were transformed (*e.g.,* MUC1 expression) may be constitutive if the promoter utilized is a constitutive promoter, or may be induced by addition of the appropriate induction conditions for the promoter if the promoter utilized is an inducible promoter (*e.g.*, copper sulfate in the case of the *CUP1* promoter). In the case of an inducible promoter, induction of protein expression may be initiated after the culture has grown to a suitable cell density, which may be at about 0.2 Y.U./ml or higher densities.

[00118] One non-limiting example of a medium suitable for the culture of a Yeast-MUC1 immunotherapy composition of the invention is U2 medium. U2 medium comprises the following components: 15g/L of glucose, 6.7 g/L of Yeast nitrogen base containing ammonium sulfate, and 0.04 mg/mL each of histidine, tryptophan, and adenine, and 0.06 mg/ml of leucine. Another non-limiting example of a medium suitable for the culture of Yeast-MUC1 immunotherapy composition of the invention is UL2 medium. UL2 medium comprises the following components: 15g/L of glucose, 6.7 g/L of Yeast nitrogen base containing ammonium sulfate, and 0.04 mg/mL each of histidine, tryptophan, and adenine.

[00119] In some embodiments of the invention, the yeast are grown under neutral pH conditions. As used herein, the general use of the term "neutral pH" refers to a pH range between about pH 5.5 and about pH 8, and in one aspect, between about pH 6 and about 8. One of skill the art will appreciate that minor fluctuations (e.g., tenths or hundredths) can occur when measuring with a pH meter. As such, the use of neutral pH to grow yeast cells means that the yeast cells are grown in neutral pH for the majority of the time that they are in culture. In one embodiment, yeast are grown in a medium maintained at a pH level of at least 5.5 (*i.e.,* the pH of the culture medium is not allowed to drop below pH 5.5). In another aspect, yeast are grown at a pH level maintained at about 6, 6.5, 7, 7.5 or 8. In one aspect, neutral pH is maintained by using a suitable buffer to create a buffered culture or growth medium. The use of a neutral pH in culturing yeast promotes several biological effects that are desirable characteristics for using the yeast as vehicles for immunomodulation. For example, culturing the yeast in neutral pH allows for good growth of the yeast without negative effect on the cell generation time (*e.g.,* slowing of doubling time). The yeast can continue to grow to high densities without losing their cell wall pliability. The use of a neutral pH allows for the production of yeast with pliable cell walls and/or yeast that are more sensitive to cell wall digesting enzymes (*e.g.,* glucanase) at all harvest densities. This trait is desirable because yeast with flexible cell walls can

induce different or improved immune responses as compared to yeast grown under more acidic conditions, *e.g.,* by promoting the secretion of cytokines by antigen presenting cells that have phagocytosed the yeast (*e.g.,* TH1-type cytokines including, but not limited to, IFN-γ, interleukin-12 (IL-12), and IL-2, as well as proinflammatory cytokines such as IL-6). In addition, greater accessibility to the antigens located in the cell wall is afforded by such culture methods. In another aspect, the use of neutral pH for some antigens allows for release of the di-sulfide bonded antigen by treatment with dithiothreitol (DTT) that is not possible when such an antigen-expressing yeast is cultured in media at lower pH (*e.g.,* pH 5). In one non-limiting example of the use of neutral pH conditions to culture yeast for use in the present invention, UL2 medium described above is buffered using, for example, 4.2g/L of Bis-Tris.

[00120] The inventors demonstrate herein that yeast-MUC1 immunotherapeutic compositions of the invention grown using neutral pH conditions are more potent activators of dendritic cells and activate MUC-1-specific T cells to produce higher levels of IFN-γ than the same yeast-MUC1 immunotherapeutic compositions grown under standard conditions (where neutral pH is not maintained) (see Examples).

[00121] In one embodiment, control of the amount of yeast glycosylation is used to control the expression of antigens by the yeast, particularly on the surface. The amount of yeast glycosylation can affect the immunogenicity and antigenicity of the antigen, particularly one expressed on the surface, since sugar moieties tend to be bulky. As such, the existence of sugar moieties on the surface of yeast and its impact on the three-dimensional space around the target antigen(s) should be considered in the modulation of yeast according to the invention. Any method can be used to reduce or increase the amount of glycosylation of the yeast, if desired. For example, one could use a yeast mutant strain that has been selected to have low glycosylation (e.g. mnn1, och1 and mnn9 mutants), or one could eliminate by mutation the glycosylation acceptor sequences on the target antigen. Alternatively, one could use yeast with abbreviated glycosylation patterns, *e.g., Pichia.* One can also treat the yeast using methods that reduce or alter the glycosylation.

[00122] In one embodiment of the present invention, as an alternative to expression of an antigen or other protein recombinantly in the yeast vehicle, a yeast vehicle is loaded intracellularly with the protein or peptide, or with carbohydrates or other molecules that serve as an antigen and/or are useful as immunomodulatory agents or biological response modifiers according to the invention. Subsequently, the yeast vehicle, which now contains

the antigen and/or other proteins intracellularly, can be administered to an individual or loaded into a carrier such as a dendritic cell. Peptides and proteins can be inserted directly into yeast vehicles of the present invention by techniques known to those skilled in the art, such as by diffusion, active transport, liposome fusion, electroporation, phagocytosis, freeze-thaw cycles and bath sonication. Yeast vehicles that can be directly loaded with peptides, proteins, carbohydrates, or other molecules include intact yeast, as well as spheroplasts, ghosts or cytoplasts, which can be loaded with antigens and other agents after production. Alternatively, intact yeast can be loaded with the antigen and/or agent, and then spheroplasts, ghosts, cytoplasts, or subcellular particles can be prepared therefrom. Any number of antigens and/or other agents can be loaded into a yeast vehicle in this embodiment, from at least 1, 2, 3, 4 or any whole integer up to hundreds or thousands of antigens and/or other agents, such as would be provided by the loading of a microorganism or portions thereof, for example.

[00123] In another embodiment of the present invention, an antigen and/or other agent is physically attached to the yeast vehicle. Physical attachment of the antigen and/or other agent to the yeast vehicle can be accomplished by any method suitable in the art, including covalent and non-covalent association methods which include, but are not limited to, chemically crosslinking the antigen and/or other agent to the outer surface of the yeast vehicle or biologically linking the antigen and/or other agent to the outer surface of the yeast vehicle, such as by using an antibody or other binding partner. Chemical cross-linking can be achieved, for example, by methods including glutaraldehyde linkage, photoaffinity labeling, treatment with carbodiimides, treatment with chemicals capable of linking di-sulfide bonds, and treatment with other cross-linking chemicals standard in the art. Alternatively, a chemical can be contacted with the yeast vehicle that alters the charge of the lipid bilayer of yeast membrane or the composition of the cell wall so that the outer surface of the yeast is more likely to fuse or bind to antigens and/or other agent having particular charge characteristics. Targeting agents such as antibodies, binding peptides, soluble receptors, and other ligands may also be incorporated into an antigen as a fusion protein or otherwise associated with an antigen for binding of the antigen to the yeast vehicle.

[00124] When the antigen or other protein is expressed on or physically attached to the surface of the yeast, spacer arms may, in one aspect, be carefully selected to optimize antigen or other protein expression or content on the surface. The size of the spacer arm(s) can affect how much of the antigen or other protein is exposed for binding on the surface

of the yeast. Thus, depending on which antigen(s) or other protein(s) are being used, one of skill in the art will select a spacer arm that effectuates appropriate spacing for the antigen or other protein on the yeast surface. In one embodiment, the spacer arm is a yeast protein of at least 450 amino acids. Spacer arms have been discussed in detail above.

[00125] In yet another embodiment, the yeast vehicle and the antigen or other protein are associated with each other by a more passive, non-specific or non-covalent binding mechanism, such as by gently mixing the yeast vehicle and the antigen or other protein together in a buffer or other suitable formulation (e.g., admixture).

[00126] In one embodiment, intact yeast (with or without expression of heterologous antigens or other proteins) can be ground up or processed in a manner to produce yeast cell wall preparations, yeast membrane particles or yeast fragments (*i.e.,* not intact) and the yeast fragments can, in some embodiments, be provided with or administered with other compositions that include antigens (*e.g.,* DNA vaccines, protein subunit vaccines, killed or inactivated pathogens, viral vector vaccines) to enhance immune responses. For example, enzymatic treatment, chemical treatment or physical force (*e.g.,* mechanical shearing or sonication) can be used to break up the yeast into parts that are used as an adjuvant.

[00127] In one embodiment of the invention, yeast vehicles useful in the invention include yeast vehicles that have been killed or inactivated. Killing or inactivating of yeast can be accomplished by any of a variety of suitable methods known in the art. For example, heat inactivation of yeast is a standard way of inactivating yeast, and one of skill in the art can monitor the structural changes of the target antigen, if desired, by standard methods known in the art. Alternatively, other methods of inactivating the yeast can be used, such as chemical, electrical, radioactive or UV methods. See, for example, the methodology disclosed in standard yeast culturing textbooks such as Methods of Enzymology, Vol. 194, Cold Spring Harbor Publishing (1990). Any of the inactivation strategies used should take the secondary, tertiary or quaternary structure of the target antigen into consideration and preserve such structure as to optimize its immunogenicity.

[00128] Yeast vehicles can be formulated into yeast-based immunotherapy compositions or products of the present invention using a number of techniques known to those skilled in the art. For example, yeast vehicles can be dried by lyophilization. Formulations comprising yeast vehicles can also be prepared by packing yeast in a cake or a tablet, such as is done for yeast used in baking or brewing operations. In addition, yeast vehicles can be mixed with a pharmaceutically acceptable excipient, such as an isotonic

buffer that is tolerated by a host or host cell. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity-enhancing agents, such as sodium carboxymethylcellulose, sorbitol, glycerol or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, m- or o-cresol, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise, for example, dextrose, human serum albumin, and/or preservatives to which sterile water or saline can be added prior to administration.

[00129] In one embodiment of the present invention, a composition can include additional agents, which may also be referred to as biological response modifier compounds, or the ability to produce such agents/modifiers. For example, a yeast vehicle can be transfected with or loaded with at least one antigen and at least one agent/biological response modifier compound, or a composition of the invention can be administered in conjunction with at least one agent/biological response modifier. Biological response modifiers include adjuvants and other compounds that can modulate immune responses, which may be referred to as immunomodulatory compounds, as well as compounds that modify the biological activity of another compound or agent, such as a yeast-based immunotherapeutic, such biological activity not being limited to immune system effects. Certain immunomodulatory compounds can stimulate a protective immune response whereas others can suppress a harmful immune response, and whether an immunomodulatory is useful in combination with a given yeast-based immunotherapeutic may depend, at least in part, on the disease state or condition to be treated or prevented, and/or on the individual who is to be treated. Certain biological response modifiers preferentially enhance a cell-mediated immune response whereas others preferentially enhance a humoral immune response (*i.e.,* can stimulate an immune response in which there is an increased level of cell-mediated compared to humoral immunity, or vice versa.). Certain biological response modifiers have one or more properties in common with the biological properties of yeast-based immunotherapeutics or enhance or complement the biological properties of yeast-based immunotherapeutics. There are a number of

techniques known to those skilled in the art to measure stimulation or suppression of immune responses, as well as to differentiate cell-mediated immune responses from humoral immune responses, and to differentiate one type of cell-mediated response from another (*e.g.,* a TH17 response versus a TH1 response).

[00130] Agents/biological response modifiers useful in the invention may include, but are not limited to, cytokines, chemokines, hormones, lipidic derivatives, peptides, proteins, polysaccharides, small molecule drugs, antibodies and antigen binding fragments thereof (including, but not limited to, anti-cytokine antibodies, anti-cytokine receptor antibodies, anti-chemokine antibodies), vitamins, polynucleotides, nucleic acid binding moieties, aptamers, and growth modulators. Some suitable agents include, but are not limited to, IL-1 or agonists of IL-1 or of IL-1R, anti-IL-1 or other IL-1 antagonists; IL-6 or agonists of IL-6 or of IL-6R, anti-IL-6 or other IL-6 antagonists; IL-12 or agonists of IL-12 or of IL-12R, anti-IL-12 or other IL-12 antagonists; IL-17 or agonists of IL-17 or of IL-17R, anti-IL-17 or other IL-17 antagonists; IL-21 or agonists of IL-21 or of IL-21R, anti-IL-21 or other IL-21 antagonists; IL-22 or agonists of IL-22 or of IL-22R, anti-IL-22 or other IL-22 antagonists; IL-23 or agonists of IL-23 or of IL-23R, anti-IL-23 or other IL-23 antagonists; IL-25 or agonists of IL-25 or of IL-25R, anti-IL-25 or other IL-25 antagonists; IL-27 or agonists of IL-27 or of IL-27R, anti-IL-27 or other IL-27 antagonists; type I interferon (including IFN-α) or agonists or antagonists of type I interferon or a receptor thereof; type II interferon (including IFN-γ) or agonists or antagonists of type II interferon or a receptor thereof; anti-CD40, CD40L, lymphocyte-activation gene 3 (LAG3) protein and/or IMP321 (T-cell immunostimulatory factor derived from the soluble form of LAG3), anti-CTLA-4 antibody (e.g., to release anergic T cells); T cell co-stimulators (e.g., anti-CD137, anti-CD28, anti-CD40); alemtuzumab (e.g., CamPath®), denileukin diftitox (e.g., ONTAK®); anti-CD4; anti-CD25; anti-PD-1, anti-PD-L1, anti-PD-L2; agents that block FOXP3 (e.g., to abrogate the activity/kill CD4+/CD25+ T regulatory cells); Flt3 ligand, imiquimod (Aldara™), granulocyte-macrophage colony stimulating factor (GM-CSF); granulocyte-colony stimulating factor (G-CSF), sargramostim (Leukine®); hormones including without limitation prolactin and growth hormone; Toll-like receptor (TLR) agonists, including but not limited to TLR-2 agonists, TLR-4 agonists, TLR-7 agonists, and TLR-9 agonists; TLR antagonists, including but not limited to TLR-2 antagonists, TLR-4 antagonists, TLR-7 antagonists, and TLR-9 antagonists; anti-inflammatory agents and immunomodulators, including but not limited to, COX-2 inhibitors (*e.g.,* Celecoxib, NSAIDS), glucocorticoids, statins, and

thalidomide and analogues thereof including IMiD™s (which are structural and functional analogues of thalidomide (*e.g.,* REVLIMID® (lenalidomide), ACTIMID® (pomalidomide)); proinflammatory agents, such as fungal or bacterial components or any proinflammatory cytokine or chemokine; immunotherapeutic vaccines including, but not limited to, virus-based vaccines, bacteria-based vaccines, or antibody-based vaccines; and any other immunomodulators, immunopotentiators, anti-inflammatory agents, pro-inflammatory agents, and any agents that modulate the number of, modulate the activation state of, and/or modulate the survival of antigen-presenting cells or of TH17, TH1, and/or Treg cells. Any combination of such agents is contemplated by the invention, and any of such agents combined with or administered in a protocol with (*e.g.*, concurrently, sequentially, or in other formats with) a yeast-based immunotherapeutic is a composition encompassed by the invention. Such agents are well known in the art. These agents may be used alone or in combination with other agents described herein.

[00131] Agents can include agonists and antagonists of a given protein or peptide or domain thereof. As used herein, an "agonist" is any compound or agent, including without limitation small molecules, proteins, peptides, antibodies, nucleic acid binding agents, etc., that binds to a receptor or ligand and produces or triggers a response, which may include agents that mimic or enhance the action of a naturally occurring substance that binds to the receptor or ligand. An "antagonist" is any compound or agent, including without limitation small molecules, proteins, peptides, antibodies, nucleic acid binding agents, etc., that blocks or inhibits or reduces the action of an agonist.

[00132] Compositions of the invention can further include or can be administered with (concurrently, sequentially, or intermittently with) any other agents or compositions or protocols that are useful for preventing or treating cancer or any compounds that treat or ameliorate any symptom of cancer, and particularly cancers associated with MUC1 expression or overexpression. In addition, compositions of the invention can be used together with other immunotherapeutic compositions, including prophylactic and/or therapeutic immunotherapy. Additional agents, compositions or protocols (*e.g.,* therapeutic protocols) that are useful for the treatment of cancer include, but are not limited to, chemotherapy, surgical resection of a tumor, radiation therapy, allogeneic or autologous stem cell transplantation, T cell adoptive transfer, other types of immunotherapy, including viral vector-based immunotherapy and dendritic cell/tumor fusion immunotherapy, and/or targeted cancer therapies (*e.g.,* small molecule drugs, biologics, or monoclonal antibody therapies that specifically target molecules involved in

tumor growth and progression, including, but not limited to, selective estrogen receptor modulators (SERMs), aromatase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone deacetylase (HDAC) inhibitors, retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, poly (ADP-ribose) polymerase (PARP) inhibitors, or immunostimulators). Any of these additional therapeutic agents and/or therapeutic protocols may be administered before, concurrently with, alternating with, or after the immunotherapy compositions of the invention, or at different time points. For example, when given to an individual in conjunction with chemotherapy or a targeted cancer therapy, it may be desirable to administer the yeast-MUC1 immunotherapy compositions during the "holiday" between doses of chemotherapy or targeted cancer therapy, in order to maximize the efficacy of the immunotherapy compositions. Surgical resection of a tumor may frequently precede administration of a yeast-MUC1 immunotherapy composition, but additional or primary surgery may occur during or after administration of a yeast-MUC1 immunotherapy composition.

[00133] The invention also includes a kit comprising any of the compositions described herein, or any of the individual components of the compositions described herein. Kits may include additional reagents and written instructions or directions for using any of the compositions of the invention to prevent or treat cancer associated with or characterized by MUC1 expression or overexpression.

Methods for Administration or Use of Compositions of the Invention

[00134] Yeast-MUC1 immunotherapeutic compositions of the invention are designed for use to prevent or treat cancers that are associated with or characterized by MUC1 expression or overexpression, including by preventing emergence of such cancers, arresting progression of such cancers or eliminating such cancers. More particularly, yeast-MUC1 immunotherapeutic compositions can be used to prevent, inhibit or delay the development of MUC1-expressing tumors, and/or to prevent, inhibit or delay tumor migration and/or tumor invasion of other tissues (metastases) and/or to generally prevent or inhibit progression of cancer in an individual. Yeast-MUC1 immunotherapeutic compositions can also be used to ameliorate at least one symptom of the cancer, such as by reducing tumor burden in the individual; inhibiting tumor growth in the individual; increasing survival of the individual; and/or preventing, inhibiting, reversing or delaying progression of the cancer in the individual.

[00135] Cancers that are relevant to the compositions and methods of the invention are any cancer that expresses, or may express, MUC1, or cancers in proximity to cancers that

express or may express MUC1, including cancers of epithelial tissues, and include, but are not limited to, cancer of the breast, small intestine, stomach, kidney, bladder, uterus, ovary, testes, lung, colon, pancreas, prostate, testes, and metastatic cancers thereof. In addition, MUC1 is or may be expressed in hematological cancers, such as lymphomas, leukemias and myelomas, including, but not limited to, chronic lymphocytic leukemia (CLL), multiple myelogenous lymphoma (MML), acute myeloid leukemia (AML), Epstein-Barr virus (EBV) transformed B cells, Burkitt's and Hodgkin's lymphomas.

[00136] In one aspect, MUC1 is not detected in the individual's cancer at the time the composition is first administered. When MUC1 is not detected in the individual's cancer, the individual may have an earlier stage cancer in which MUC1 expression has not yet manifested (*e.g.,* stage I or stage II), or in which MUC1 expression is not yet detectable in any event (*i.e.,* MUC1 may or may not be expressed at a low level or in a small number of tumor cells, but is not yet readily detectable using standard detection methods). Alternatively, the individual may have precancerous lesions or tumors, or may be known to be predisposed to developing cancer (*e.g.,* by knowledge of family history, genetic markers, etc.). In these aspects of the invention, the development of MUC1-expressing tumor cells is prevented, delayed or inhibited by use of the Yeast-MUC1 immunotherapeutic composition.

[00137] In another aspect, MUC1 expression is or can be detected in the individual's cancer at the time the composition is first administered. The individual may have stage I, stage II, stage III, or stage IV cancer in this aspect of the invention. In this aspect, use of the Yeast-MUC1 immunotherapeutic composition reduces, eliminates or slows or arrests the growth of tumors expressing MUC1, which can result in reduction in tumor burden in the individual, inhibition of MUC1-expressing tumor growth, and/or increased survival of the individual.

[00138] Another embodiment of the invention relates to a method to treat cancer, and particularly, a MUC1-expressing cancer. The method includes administering to an individual who has a MUC1-expressing cancer a Yeast-MUC1 immunotherapeutic composition described herein, which can include a composition comprising: (a) a yeast vehicle; and (b) a cancer antigen comprising at least one MUC1 antigen. In one aspect, the method reduces tumor burden in the patient. In one aspect, the method increases survival of the patient. In one aspect, the method inhibits tumor growth in the individual.

[00139] In one aspect, the individual is additionally treated with at least one other therapeutic compound or therapeutic protocol useful for the treatment of cancer. Such

therapeutic agents and protocols have been discussed in detail elsewhere herein. For example, in any of the embodiments regarding methods of the invention described herein, in one aspect, when the individual has cancer (regardless of the status of detectable MUC1 expression in tumor cells) the individual is being treated or has been treated with another therapy for cancer. Such therapy can include any of the therapeutic protocols or use of any therapeutic compound or agent described previously herein, including, but not limited to, chemotherapy, radiation therapy, targeted cancer therapy, surgical resection of a tumor, stem cell transfer, cytokine therapy, adoptive T cell transfer, and/or administration of a second immunotherapeutic composition. In the case of administration of a second immunotherapeutic composition, such compositions may include, but are not limited to, additional yeast-based immunotherapy, recombinant virus-based immunotherapy (viral vectors, *e.g.,* see PCT Publication No. WO/00/34494), cytokine therapy, immunostimulant therapy (including chemotherapy with immunostimulating properties), DNA vaccines, dendritic cell/tumor fusion immunotherapy (*e.g.,* see PCT Publication No. WO/2009/062001), and other immunotherapy compositions.

[00140] In one aspect, the second immunotherapeutic composition includes a second cancer antigen that is not a MUC1 antigen. For example, a second immunotherapeutic composition useful in combination with a Yeast-MUC1 immunotherapeutic composition is a yeast-immunotherapeutic composition comprising another cancer antigen that is expressed by the same tumor type, or by other tumors the individual to be treated has or may develop. Such cancer antigens include, but are not limited to, any one or more of carcinoembryonic antigen (CEA), point mutated Ras oncoprotein, Brachyury, EGFR, BCR-Abl, MART-1, MAGE-1, MAGE-3, GAGE, GP-100, MUC-2, normal and point mutated p53 oncoproteins, PSMA, tyrosinase, TRP-1 (gp75), NY-ESO-1, TRP-2, TAG72, KSA, CA-125, PSA, HER-2/neu/c-erb/B2, hTERT, p73, B-RAF, adenomatous polyposis coli (APC), Myc, von Hippel-Lindau protein (VHL), Rb-1, Rb-2, androgen receptor (AR), Smad4, MDR1, Flt-3, BRCA-1, BRCA-2, pax3-fkhr, ews-fli-1, HERV-H, HERV-K, TWIST, Mesothelin, NGEP, modifications of such antigens, splice variants of such antigens, and epitope agonists of such antigens, as well as combinations of such antigens, and/or immunogenic domains thereof, modifications thereof, variants thereof, and/or epitope agonists thereof.

[00141] As used herein, to "treat" a cancer, or any permutation thereof (*e.g.,* "treated for cancer", etc.) generally refers to administering a composition of the invention once the cancer has occurred (*e.g.,* once the cancer has been diagnosed or detected in an individual),

with at least one therapeutic goal of the treatment (as compared to in the absence of this treatment) including: reduction in tumor burden; inhibition of tumor growth; increase in survival of the individual; delaying, inhibiting, arresting or preventing the onset or development of metastatic cancer (such as by delaying, inhibiting, arresting or preventing the onset of development of tumor migration and/or tumor invasion of tissues outside of primary cancer and/or other processes associated with metastatic progression of cancer); delaying or arresting primary cancer progression; improvement of immune responses against the tumor; improvement of long term memory immune responses against the tumor antigens,; and/or improved general health of the individual. To "prevent" or "protect" from a cancer, or any permutation thereof (*e.g.,* "prevention of cancer", etc.), generally refers to administering a composition of the invention before a cancer has occurred, when pre-cancerous cells are detected, or before a specific stage of cancer or tumor antigen expression in a cancer has occurred (*e.g.,* before MUC1 expression is detected in the cancer), with at least one goal of the treatment (as compared to in the absence of this treatment) including: preventing or delaying the onset or development of a cancer, or, should the cancer occur after the treatment, at least reducing the severity of the cancer (*e.g.,* reducing the level of tumor growth, arresting cancer progression, improving the immune response against the cancer, inhibiting metastatic processes, etc.) or improving outcomes in the individual (*e.g.,* improving survival).

[00142] The present invention includes the delivery (administration, immunization, vaccination) of a Yeast-MUC1 immunotherapeutic composition of the invention to a subject or individual. The administration process can be performed *ex vivo* or *in vivo*, but is typically performed *in vivo*. *Ex vivo* administration refers to performing part of the regulatory step outside of the patient, such as administering a composition of the present invention to a population of cells (dendritic cells) removed from a patient under conditions such that a yeast vehicle, antigen(s) and any other agents or compositions are loaded into the cell, and returning the cells to the patient. The therapeutic composition of the present invention can then be returned to a patient, or administered to a patient, by any suitable mode of administration.

[00143] Administration of a composition can be systemic, mucosal and/or proximal to the location of the target site (*e.g.,* near a site of a tumor). Suitable routes of administration will be apparent to those of skill in the art, depending on the type of cancer to be prevented or treated and/or the target cell population or tissue. Various acceptable methods of administration include, but are not limited to, intravenous administration,

intraperitoneal administration, intramuscular administration, intranodal administration, intracoronary administration, intraarterial administration (*e.g.,* into a carotid artery), subcutaneous administration, transdermal delivery, intratracheal administration, intraarticular administration, intraventricular administration, inhalation (*e.g.*, aerosol), intracranial, intraspinal, intraocular, aural, intranasal, oral, pulmonary administration, impregnation of a catheter, and direct injection into a tissue. In one aspect, routes of administration include: intravenous, intraperitoneal, subcutaneous, intradermal, intranodal, intramuscular, transdermal, inhaled, intranasal, oral, intraocular, intraarticular, intracranial, and intraspinal. Parenteral delivery can include intradermal, intramuscular, intraperitoneal, intrapleural, intrapulmonary, intravenous, subcutaneous, atrial catheter and venal catheter routes. Aural delivery can include ear drops, intranasal delivery can include nose drops or intranasal injection, and intraocular delivery can include eye drops. Aerosol (inhalation) delivery can also be performed using methods standard in the art (see, for example, Stribling et al., *Proc. Natl. Acad. Sci. USA* 189:11277-11281, 1992). In one aspect, a Yeast-MUC1 immunotherapeutic composition of the invention is administered subcutaneously. In one aspect, the Yeast-MUC1 immunotherapeutic composition is administered directly into a tumor milieu.

[00144] In general, a suitable single dose of a Yeast-MUC1 immunotherapeutic composition is a dose that is capable of effectively providing a yeast vehicle and the MUC1 antigen to a given cell type, tissue, or region of the patient body in an amount effective to elicit an antigen-specific immune response against one or more MUC1 antigens or epitopes, when administered one or more times over a suitable time period. For example, in one embodiment, a single dose of a Yeast-MUC1 of the present invention is from about 1×10^5 to about 5×10^7 yeast cell equivalents per kilogram body weight of the organism being administered the composition. One Yeast Unit (Y.U.) is 1×10^7 yeast cells or yeast cell equivalents. In one aspect, a single dose of a yeast vehicle of the present invention is from about 0.1 Y.U. (1×10^6 yeast cells or yeast cell equivalents) to about 100 Y.U. (1×10^9 cells) per dose (*i.e.,* per organism), including any interim dose, in increments of 0.1×10^6 cells (i.e., 1.1×10^6, 1.2×10^6, 1.3×10^6...). In one embodiment, a suitable dose includes doses between 1 Y.U. and 40 Y.U. and in one aspect, between 10 Y.U. and 40 Y.U. or between 10 Y.U. and 80 Y.U. In one embodiment, the doses are administered at different sites on the individual but during the same dosing period. For example, a 40 Y.U. dose may be administered by injecting 10 Y.U. doses to four different sites on the individual during one dosing period. The invention includes administration of an amount

of the Yeast-MUC1 immunotherapy composition (*e.g.,* 1, 2, 3, 4, 5, 6, 7, 8, 9 10, 11, 12, 13, 14,15, 16, 17, 18, 19, 20 Y.U. or more) at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different sites on an individual to form a single dose.

[00145] "Boosters" or "boosts" of a therapeutic composition are administered, for example, when the immune response against the antigen has waned or as needed to provide an immune response or induce a memory response against a particular antigen or antigen(s). Boosters can be administered about 1, 2, 3, 4, 5, 6, 7, or 8 weeks apart, or monthly, bimonthly, quarterly, annually, and/or in a few or several year increments after the original administration, depending on the status of the individual being treated and the goal of the therapy at the time of administration (*e.g.,* prophylactic, active treatment, maintenance). In one embodiment, an administration schedule is one in which doses of Yeast-MUC1 immunotherapeutic composition is administered at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times over a time period of from weeks, to months, to years. In one embodiment, the doses are administered weekly or biweekly for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more doses, followed by biweekly or monthly doses as needed to achieve the desired preventative or therapeutic treatment for cancer. Additional boosters can then be given at similar or longer intervals (months or years) as a maintenance or remission therapy, if desired.

[00146] In one aspect of the invention, one or more additional therapeutic agents or therapeutic protocols are administered or performed sequentially and/or concurrently with the administration of the Yeast-MUC1 immunotherapy composition, *e.g.,* surgical resection of the tumor, administration of chemotherapy, administration of radiation therapy, administration of another immunotherapy composition or protocol including viral vector therapy and dendritic cell/tumor fusion therapy, cytokine therapy, adoptive T cell transfer (including adoptive transfer of T cells that have been stimulated *ex vivo* by an antigen and/or immunotherapy composition), or stem cell transplantation. In one example, yeast-MUC1 immunotherapy is administered in conjunction with a therapy utilizing viral vector-based immunotherapy, such as that described in PCT Publication No. WO/00/34494. In another example, yeast-MUC1 immunotherapy is administered in conjunction with dendritic cell/tumor cell fusion therapy or immune system cells (*e.g.,* T cells) stimulated with such dendritic cell/tumor cell fusions, such as that described in PCT Publication No. WO/2009062001. In such embodiments, the non-yeast-based immunotherapy may target the MUC1 or a different tumor antigen, and such therapies

may include the additional administration of other agents, such as cytokines, antibodies, or other agents.

[00147] In one aspect, one or more therapies for cancer (including any therapies described herein or otherwise known in the art) can be administered or performed prior to the first dose of Yeast-MUC1 immunotherapy composition or after the first dose is administered. In one embodiment, one or more therapies can be administered or performed in an alternating manner with the dosing of Yeast-MUC1 immunotherapy composition, such as in a protocol in which the Yeast-MUC1 composition is administered at prescribed intervals in between one or more consecutive doses of chemotherapy or other therapy. In one embodiment, the Yeast-MUC1 immunotherapy composition is administered in one or more doses over a period of time prior to commencing additional therapies. In other words, the Yeast-MUC1 immunotherapeutic composition is administered as a monotherapy for a period of time, and then an additional therapy is added (*e.g.,* chemotherapy), either concurrently with new doses of Yeast-MUC1 immunotherapy, or in an alternating fashion with Yeast-MUC1 immunotherapy. Alternatively or in addition, another therapy may be administered for a period of time prior to beginning administration of the Yeast-MUC1 immunotherapy composition, and the concepts may be combined (*e.g.,* surgical resection of a tumor, followed by monotherapy with Yeast-MUC1 immunotherapy for several weeks, followed by alternating doses of chemotherapy and Yeast-MUC1 immunotherapy for weeks or months, optionally followed by monotherapy using Yeast-MUC1 immunotherapy and/or another therapy, or by a new protocol of combinations of therapy provided sequentially, concurrently, or in alternating fashion). Various protocols for the treatment of cancer using Yeast-MUC1 immunotherapy are contemplated by the invention, and these examples should be considered to be non-limiting examples of various possible protocols.

[00148] A virus-based immunotherapy composition typically comprises a viral vector comprising a virus genome or portions thereof (*e.g.,* a recombinant virus) and a nucleic acid sequence encoding at least one antigen(s) from a disease causing agent or disease state (*e.g.,* a cancer antigen(s), infectious disease antigen(s), and/or at least one immunogenic domain thereof). In some embodiments, a virus-based immunotherapy composition further includes at least one viral vector comprising one or more nucleic acid sequences encoding one or more immunostimulatory molecule(s). In some embodiments, the genes encoding immunostimulatory molecules and antigens are inserted into the same viral vector (the same recombinant virus).

[00149] Dendritic cell/tumor cell fusion immunotherapy compositions typically are hybrid cells generated by the fusion between dendritic cells and non-dendritic cells that express tumor antigens, including tumor cells, using fusion methods that are known in the art. The fused cells have dendritic cell characteristics and also express and present tumor antigens from the tumor cell. The compositions may be administered to an individual, or used to stimulate T cells *ex vivo* for T cell transfer methods.

[00150] In one aspect of the invention, additional antigens other than MUC1 are also targeted using yeast-based immunotherapy, in addition to targeting MUC1. Such additional target antigens can be included within the same yeast-vehicle as the MUC1 antigens, or additional yeast-based immunotherapy compositions targeting different antigens can be produced and then combined as desired depending on the individual to be treated, the antigens expressed by the type of cancer or by the individual's particular tumor, and/or depending on the stage of cancer in the individual, or the stage of treatment of the individual. For examples a combination of antigens may be selected that cover: (1) antigens involved in seminal events in cancer development, such as mutated Ras, (2) antigens involved in or associated with dysregulation of cellular processes, such as CEA or MUC1, and (3) Brachyury, which is involved in metastatic processes. For example, one or more other yeast-based immunotherapy compositions may express one or more antigens including, but not limited to, carcinoembryonic antigen (CEA), point mutated Ras oncoprotein, Brachyury, EGFR, BCR-Abl, MART-1, MAGE-1, MAGE-3, GAGE, GP-100, MUC-2, normal and point mutated p53 oncoproteins, PSMA, tyrosinase, TRP-1 (gp75), NY-ESO-1, TRP-2, TAG72, KSA, CA-125, PSA, HER-2/neu/c-erb/B2, hTERT, p73, B-RAF, adenomatous polyposis coli (APC), Myc, von Hippel-Lindau protein (VHL), Rb-1, Rb-2, androgen receptor (AR), Smad4, MDR1, Flt-3, BRCA-1, BRCA-2, pax3-fkhr, ews-fli-1, HERV-H, HERV-K, TWIST, Mesothelin, NGEP, modifications of such antigens, splice variants of such antigens, and epitope agonists of such antigens, as well as combinations of such antigens, and/or immunogenic domains thereof, modifications thereof, variants thereof, and/or epitope agonists thereof. One, two, three, or more of these yeast-based immunotherapy compositions may be administered to an individual prior to, concurrently or alternating with, and/or after administration of a Yeast-MUC1 immunotherapy composition, in order to optimize targeting of antigens in the individual's tumor. As above, additional therapies can also be used in such protocols (*e.g.,* surgical resection of tumor, chemotherapy, targeted cancer therapy, radiation therapy, etc.).

[00151] In one embodiment of the invention, a method to treat cancer is provided. The method includes the steps of: (a) administering to an individual who has cancer or pre-cancerous tumor, a first immunotherapeutic composition comprising a yeast vehicle and a MUC1 antigen as described herein; and (b) administering to the individual, prior to, concurrently with, or subsequent to, administration of the first immunotherapeutic composition one or more additional immunotherapeutic compositions, each comprising a yeast vehicle and each comprising a different cancer antigen that is not a MUC1 antigen. The additional cancer antigen can be any of those known in the art or described herein, including, but not limited to, mutated Ras, carcinoembryonic antigen (CEA), Brachyury, EGFR, etc. Steps may be repeated as needed to treat a particular individual's cancer, and the cancer antigens can be modified before or during treatment to specifically address the particular individual's cancer.

[00152] In the method of the present invention, compositions and therapeutic compositions can be administered to any animal, including any vertebrate, and particularly to any member of the Vertebrate class, *Mammalia*, including, without limitation, primates, rodents, livestock and domestic pets. Livestock include mammals to be consumed or that produce useful products (e.g., sheep for wool production). Mammals to treat or protect utilizing the invention include humans, non-human primates, dogs, cats, mice, rats, goats, sheep, cattle, horses and pigs.

[00153] An "individual" is a vertebrate, such as a mammal, including without limitation a human. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, mice and rats. The term "individual" can be used interchangeably with the term "animal", "subject" or "patient".

General Techniques Useful in the Invention

[00154] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are well known to those skilled in the art. Such techniques are explained fully in the literature, such as, Methods of Enzymology, Vol. 194, Guthrie et al., eds., Cold Spring Harbor Laboratory Press (1990); Biology and activities of yeasts, Skinner, et al., eds., Academic Press (1980); Methods in yeast genetics : a laboratory course manual, Rose et al., Cold Spring Harbor Laboratory Press (1990); The Yeast Saccharomyces: Cell Cycle and Cell Biology, Pringle et al., eds., Cold Spring Harbor Laboratory Press (1997); The Yeast Saccharomyces: Gene Expression, Jones et al., eds., Cold Spring Harbor Laboratory

Press (1993); The Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics, Broach et al., eds., Cold Spring Harbor Laboratory Press (1992); Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) and Molecular Cloning: A Laboratory Manual, third edition (Sambrook and Russel, 2001), (jointly referred to herein as "Sambrook"); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987, including supplements through 2001); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Harlow and Lane (1988), Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York; Harlow and Lane (1999) Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (jointly referred to herein as "Harlow and Lane"), Beaucage et al. eds., Current Protocols in Nucleic Acid Chemistry, John Wiley & Sons, Inc., New York, 2000); Casarett and Doull's Toxicology The Basic Science of Poisons, C. Klaassen, ed., 6th edition (2001), and Vaccines, S. Plotkin, W. Orenstein, and P. Offit, eds., Fifth Edition (2008).

General Definitions

[00155] A "TARMOGEN®" (GlobeImmune, Inc., Louisville, Colorado) generally refers to a yeast vehicle expressing one or more heterologous antigens extracellularly (on its surface), intracellularly (internally or cytosolically) or both extracellularly and intracellularly. TARMOGEN®s have been generally described (see, *e.g.,* U.S. Patent No. 5,830,463). Certain yeast-based immunotherapy compositions, and methods of making and generally using the same, are also described in detail, for example, in U.S. Patent No. 5,830,463, U.S. Patent No. 7,083,787, U.S. Patent No. 7,736,642, Stubbs et al., *Nat. Med.* 7:625-629 (2001), Lu et al., *Cancer Research* 64:5084-5088 (2004), and in Bernstein et al., *Vaccine* 2008 Jan 24;26(4):509-21, each of which is incorporated herein by reference in its entirety.

[00156] As used herein, the term "analog" refers to a chemical compound that is structurally similar to another compound but differs slightly in composition (as in the replacement of one atom by an atom of a different element or in the presence of a particular functional group, or the replacement of one functional group by another functional group). Thus, an analog is a compound that is similar or comparable in function and appearance, but has a different structure or origin with respect to the reference compound.

[00157] The terms "substituted", "substituted derivative" and "derivative", when used to describe a compound, means that at least one hydrogen bound to the unsubstituted compound is replaced with a different atom or a chemical moiety.

[00158] Although a derivative has a similar physical structure to the parent compound, the derivative may have different chemical and/or biological properties than the parent compound. Such properties can include, but are not limited to, increased or decreased activity of the parent compound, new activity as compared to the parent compound, enhanced or decreased bioavailability, enhanced or decreased efficacy, enhanced or decreased stability *in vitro* and/or *in vivo*, and/or enhanced or decreased absorption properties.

[00159] In general, the term "biologically active" indicates that a compound (including a protein or peptide) has at least one detectable activity that has an effect on the metabolic, physiological, chemical, or other processes of a cell, a tissue, or an organism, as measured or observed *in vivo* (*i.e.,* in a natural physiological environment) or *in vitro* (*i.e.,* under laboratory conditions).

[00160] According to the present invention, the term "modulate" can be used interchangeably with "regulate" and refers generally to upregulation or downregulation of a particular activity. As used herein, the term "upregulate" can be used generally to describe any of: elicitation, initiation, increasing, augmenting, boosting, improving, enhancing, amplifying, promoting, or providing, with respect to a particular activity. Similarly, the term "downregulate" can be used generally to describe any of: decreasing, reducing, inhibiting, ameliorating, diminishing, lessening, blocking, or preventing, with respect to a particular activity.

[00161] In one embodiment of the present invention, any of the amino acid sequences described herein can be produced with from at least one, and up to about 20, additional heterologous amino acids flanking each of the C- and/or N-terminal ends of the specified amino acid sequence. The resulting protein or polypeptide can be referred to as "consisting essentially of" the specified amino acid sequence. According to the present invention, the heterologous amino acids are a sequence of amino acids that are not naturally found (*i.e.,* not found in nature, in vivo) flanking the specified amino acid sequence, or that are not related to the function of the specified amino acid sequence, or that would not be encoded by the nucleotides that flank the naturally occurring nucleic acid sequence encoding the specified amino acid sequence as it occurs in the gene, if such nucleotides in the naturally occurring sequence were translated using standard codon

usage for the organism from which the given amino acid sequence is derived. Similarly, the phrase "consisting essentially of", when used with reference to a nucleic acid sequence herein, refers to a nucleic acid sequence encoding a specified amino acid sequence that can be flanked by from at least one, and up to as many as about 60, additional heterologous nucleotides at each of the 5' and/or the 3' end of the nucleic acid sequence encoding the specified amino acid sequence. The heterologous nucleotides are not naturally found (*i.e.,* not found in nature, *in vivo*) flanking the nucleic acid sequence encoding the specified amino acid sequence as it occurs in the natural gene or do not encode a protein that imparts any additional function to the protein or changes the function of the protein having the specified amino acid sequence.

[00162] According to the present invention, the phrase "selectively binds to" refers to the ability of an antibody, antigen-binding fragment or binding partner of the present invention to preferentially bind to specified proteins. More specifically, the phrase "selectively binds" refers to the specific binding of one protein to another (*e.g.,* an antibody, fragment thereof, or binding partner to an antigen), wherein the level of binding, as measured by any standard assay (*e.g.,* an immunoassay), is statistically significantly higher than the background control for the assay. For example, when performing an immunoassay, controls typically include a reaction well/tube that contain antibody or antigen binding fragment alone (*i.e.,* in the absence of antigen), wherein an amount of reactivity (*e.g.*, non-specific binding to the well) by the antibody or antigen-binding fragment thereof in the absence of the antigen is considered to be background. Binding can be measured using a variety of methods standard in the art including enzyme immunoassays (*e.g.*, ELISA, immunoblot assays, etc.).

[00163] General reference to a protein or polypeptide used in the present invention includes full-length proteins, or any fragment, domain (structural, functional, or immunogenic), conformational epitope, or a homologue or variant of a given protein. A fusion protein may also be generally referred to as a protein or polypeptide. An isolated protein, according to the present invention, is a protein (including a polypeptide or peptide) that has been removed from its natural milieu (*i.e.,* that has been subject to human manipulation) and can include purified proteins, partially purified proteins, recombinantly produced proteins, and synthetically produced proteins, for example. As such, "isolated" does not reflect the extent to which the protein has been purified. Preferably, an isolated protein of the present invention is produced recombinantly. According to the present invention, the terms "modification" and "mutation" can be used interchangeably,

particularly with regard to the modifications/mutations to the amino acid sequence of proteins or portions thereof (or nucleic acid sequences) described herein.

[00164] As used herein, the term "homologue" or "variant" is used to refer to a protein or peptide which differs from a reference protein or peptide (*i.e.*, the "prototype" or "wild-type" protein) by minor modifications to the reference protein or peptide, but which maintains the basic protein and side chain structure of the naturally occurring form. Such changes include, but are not limited to: changes in one or a few amino acid side chains; changes one or a few amino acids, including deletions (*e.g.*, a truncated version of the protein or peptide) insertions and/or substitutions; changes in stereochemistry of one or a few atoms; and/or minor derivatizations, including but not limited to: methylation, glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation and/or addition of glycosylphosphatidyl inositol. A homologue or variant can have enhanced, decreased, or substantially similar properties as compared to the reference protein or peptide. A homologue or variant can include an agonist of a protein or an antagonist of a protein. Homologues or variants can be produced using techniques known in the art for the production of proteins including, but not limited to, direct modifications to the isolated reference protein, direct protein synthesis, or modifications to the nucleic acid sequence encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis, resulting in the encoding of a protein variant. In addition, naturally occurring variants of a reference protein may exist (*e.g.,* isoforms, allelic variants, or other natural variants that may occur from individual to individual) and may be isolated, produced and/or utilized in the invention.

[00165] A homologue or variant of a given protein may comprise, consist essentially of, or consist of, an amino acid sequence that is at least about 45%, or at least about 50%, or at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 86% identical, or at least about 87% identical, or at least about 88% identical, or at least about 89% identical, or at least about 90%, or at least about 91% identical, or at least about 92% identical, or at least about 93% identical, or at least about 94% identical, or at least about 95% identical, or at least about 96% identical, or at least about 97% identical, or at least about 98% identical, or at least about 99% identical (or any percent identity between 45% and 99%, in whole integer increments), to the amino acid sequence of the reference protein (*e.g.,* an amino acid sequence specified herein, or the amino acid sequence of a specified protein). In one embodiment, the homologue or variant comprises, consists

essentially of, or consists of, an amino acid sequence that is less than 100% identical, less than about 99% identical, less than about 98% identical, less than about 97% identical, less than about 96% identical, less than about 95% identical, and so on, in increments of 1%, to less than about 70% identical to the amino acid sequence of the reference protein.

[00166] As used herein, unless otherwise specified, reference to a percent (%) identity refers to an evaluation of homology which is performed using: (1) a Basic Local Alignment Search Tool (BLAST) basic homology search using blastp for amino acid searches and blastn for nucleic acid searches with standard default parameters, wherein the query sequence is filtered for low complexity regions by default (such as described in Altschul, S.F., Madden, T.L., Schääffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res.* 25:3389-3402, incorporated herein by reference in its entirety); (2) a BLAST alignment of two sequences (*e.g.,* using the parameters described below); (3) and/or PSI-BLAST with the standard default parameters (Position-Specific Iterated BLAST. It is noted that due to some differences in the standard parameters between Basic BLAST and BLAST for two sequences, two specific sequences might be recognized as having significant homology using the BLAST program, whereas a search performed in Basic BLAST using one of the sequences as the query sequence may not identify the second sequence in the top matches. In addition, PSI-BLAST provides an automated, easy-to-use version of a "profile" search, which is a sensitive way to look for sequence homologues. The program first performs a gapped BLAST database search. The PSI-BLAST program uses the information from any significant alignments returned to construct a position-specific score matrix, which replaces the query sequence for the next round of database searching. Therefore, it is to be understood that percent identity can be determined by using any one of these programs.

[00167] Two specific sequences can be aligned to one another using BLAST as described in Tatusova and Madden, (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250, incorporated herein by reference in its entirety. Such a sequence alignment is performed in blastp or blastn using the BLAST 2.0 algorithm to perform a Gapped BLAST search (BLAST 2.0) between the two sequences allowing for the introduction of gaps (deletions and insertions) in the resulting alignment. For purposes of clarity herein, a BLAST sequence alignment for two sequences is performed using the standard default parameters as follows.

For blastn, using 0 BLOSUM62 matrix:

Reward for match = 1
Penalty for mismatch = -2
Open gap (5) and extension gap (2) penalties
gap x_dropoff (50) expect (10) word size (11) filter (on)

For blastp, using 0 BLOSUM62 matrix:
Open gap (11) and extension gap (1) penalties
gap x_dropoff (50) expect (10) word size (3) filter (on).

[00168] An isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (*i.e.*, that has been subject to human manipulation), its natural milieu being the genome or chromosome in which the nucleic acid molecule is found in nature. As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has been purified, but indicates that the molecule does not include an entire genome or an entire chromosome or a segment of the genome containing more than one gene, in which the nucleic acid molecule is found in nature. An isolated nucleic acid molecule can include a complete gene. An isolated nucleic acid molecule that includes a gene is not a fragment of a chromosome that includes such gene, but rather includes the coding region and regulatory regions associated with the gene, but no additional genes that are naturally found on the same chromosome. An isolated nucleic acid molecule may also include portions of a gene. An isolated nucleic acid molecule can also include a specified nucleic acid sequence flanked by (*i.e.*, at the 5' and/or the 3' end of the sequence) additional nucleic acids that do not normally flank the specified nucleic acid sequence in nature (*i.e.*, heterologous sequences). Isolated nucleic acid molecule can include DNA, RNA (*e.g.*, mRNA), or derivatives of either DNA or RNA (*e.g.,* cDNA). Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a protein or domain of a protein.

[00169] A recombinant nucleic acid molecule is a molecule that can include at least one of any nucleic acid sequence encoding any one or more proteins described herein operatively linked to at least one of any transcription control sequence capable of effectively regulating expression of the nucleic acid molecule(s) in the cell to be transfected. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used

interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a protein. In addition, the phrase "recombinant molecule" primarily refers to a nucleic acid molecule operatively linked to a transcription control sequence, but can be used interchangeably with the phrase "nucleic acid molecule" which is administered to an animal.

[00170] A recombinant nucleic acid molecule includes a recombinant vector, which is any nucleic acid sequence, typically a heterologous sequence, which is operatively linked to the isolated nucleic acid molecule encoding a fusion protein of the present invention, which is capable of enabling recombinant production of the fusion protein, and which is capable of delivering the nucleic acid molecule into a host cell according to the present invention. Such a vector can contain nucleic acid sequences that are not naturally found adjacent to the isolated nucleic acid molecules to be inserted into the vector. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and preferably in the present invention, is a plasmid useful for transfecting yeast. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of nucleic acid molecules, and can be used in delivery of such molecules (*e.g.*, as in a DNA composition or a viral vector-based composition). Recombinant vectors are preferably used in the expression of nucleic acid molecules, and can also be referred to as expression vectors. Preferred recombinant vectors are capable of being expressed in a transfected host cell, such as a yeast.

[00171] In a recombinant molecule of the present invention, nucleic acid molecules are operatively linked to expression vectors containing regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the host cell and that control the expression of nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include nucleic acid molecules that are operatively linked to one or more expression control sequences. The phrase "operatively linked" refers to linking a nucleic acid molecule to an expression control sequence in a manner such that the molecule is expressed when transfected (i.e., transformed, transduced or transfected) into a host cell.

[00172] According to the present invention, the term “transfection” is used to refer to any method by which an exogenous nucleic acid molecule (i.e., a recombinant nucleic acid molecule) can be inserted into a cell. The term "transformation" can be used interchangeably with the term "transfection" when such term is used to refer to the introduction of nucleic acid molecules into microbial cells, such as algae, bacteria and

yeast. In microbial systems, the term "transformation" is used to describe an inherited change due to the acquisition of exogenous nucleic acids by the microorganism and is essentially synonymous with the term "transfection." Therefore, transfection techniques include, but are not limited to, transformation, chemical treatment of cells, particle bombardment, electroporation, microinjection, lipofection, adsorption, infection and protoplast fusion.

[00173] The following experimental results are provided for purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

[00174] The following example describes the production of a Yeast-MUC1 immunotherapeutic composition known as GI-6101.

[00175] In this experiment, yeast (*Saccharomyces cerevisiae*) were engineered to express a human MUC1 antigen under the control of the copper-inducible promoter, *CUP1*, or the constitutive promoter, *TEF2*, producing yeast-MUC1 immunotherapy compositions. In each case, a fusion protein comprising a MUC1 antigen was produced as a single polypeptide with the following sequence elements fused in frame from N- to C-terminus, represented by SEQ ID NO:15: (1) a MUC1 SEA/ED segment (positions 1-59 of SEQ ID NO:15); (2) a VNTR segment comprising two VNTR domains (positions 60-100 of SEQ ID NO:15); (3) a MUC1 TM domain (positions 101-128 of SEQ ID NO:15); and (4) a MUC1 CD (positions 129-200 of SEQ ID NO:15). This fusion protein further included a MUC1 signal sequence (positions 1-30 of SEQ ID NO:14) that could be substituted with a different N-terminal sequence designed to impart resistance to proteasomal degradation and/or stabilize expression, such as the peptide represented by SEQ ID NO:21, or an N-terminal peptide from a yeast alpha leader sequence such as SEQ ID NO:19 or SEQ ID NO:20. The complete fusion protein including the N-terminal MUC1 signal sequence and a hexahistidine C-terminal tag to facilitate identification and/or purification of the protein is a single polypeptide with the following sequence elements fused in frame from N- to C-terminus, represented by SEQ ID NO:14: (1) MUC1 signal sequence (positions 1-30 of SEQ ID NO:14); (2) a MUC1 SEA/ED segment (positions 31-89 of SEQ ID NO:14); (3) a VNTR segment comprising two VNTR domains (positions 90-130 of SEQ ID NO:14); (4) a MUC1 TM domain (positions 131-158 of SEQ ID NO:14); (5) a MUC1 CD (positions 159-230 of SEQ ID NO:14); and (6) a hexapeptide histidine tag (positions 231-236 of SEQ ID NO:14). SEQ ID NO:14 is

encoded by the nucleotide sequence represented by SEQ ID NO:13 (codon optimized for yeast expression).

[00176] Briefly, DNA encoding the MUC1 antigens were synthesized, and then inserted at *EcoRI* and *NotI* cloning sites behind the *CUP1* promoter (vector pGI-100) or the *TEF2* promoter (vector plu011) in yeast 2 μm expression vectors. Nucleotide sequences encoding a C-terminal hexahistidine peptide were added to the plasmid vector to encode the complete fusion protein represented by SEQ ID NO:14. The resulting plasmids were transformed into DH5α for plasmid storage, and into *Saccharomyces cerevisiae* W303α for production of the yeast-MUC1 immunotherapeutic compositions.

[00177] Transformation into *Saccharomyces cerevisiae* was performed by lithium acetate/polyethylene glycol transfection, and primary transfectants were selected on solid minimal plates lacking Uracil (UDM; uridine dropout medium). Colonies were selected by growing in U2 (uridine dropout medium) or UL2 (uridine and leucine dropout medium) medium at 30° C.

[00178] The yeast-MUC1 immunotherapy composition comprising a polynucleotide encoding the human MUC1 fusion protein represented by SEQ ID NO:14 under the control of the *TEF2* promoter is also referred to herein as GI-6101.

[00179] Liquid cultures lacking uridine (U2) or lacking uridine and leucine (UL2) were inoculated using the plates and starter cultures described above, and were grown for about 24h at 30°C, 250 rpm. pH buffered UL2 medium containing 4.2g/L of Bis-Tris (BT-UL2) was also inoculated from frozen yeast banks to evaluate yeast-MUC1 immunotherapeutics produced under neutral pH manufacturing conditions (data not shown). Culturing in pH buffered UL2 medium exposes β-glucans on the yeast cell wall and is believed to modify the cellular immune responses induced by the yeast as a result of modifying the interactions with dectin receptors on antigen presenting cells. The remaining culture conditions were the same whether U2, UL2 or BT-UL2 was used. Primary cultures were used to inoculate final cultures of the same formulation.

Recipe for U2 liquid media:

15g/L of glucose

6.7 g/L of Yeast nitrogen base containing ammonium sulfate

0.04 g/L each of histidine, tryptophan, adenine and 0.06 g/L of leucine

Recipe for UL2 liquid media:

15g/L of glucose

6.7 g/L of Yeast nitrogen base containing ammonium sulfate

0.04 g/L each of histidine, tryptophan, and adenine

[00180] In initial experiments comparing yeast-MUC1 immunotherapeutic compositions under the control of different promoters, *CUP1*-driven (inducible expression) yeast-MUC1 expression was produced in a 2-step or 3-step culture; after starter or intermediate culture reaches mid-log (1.5-4 Y.U./ml), the expression was initiated by the addition of 0.375 mM copper sulfate to final the culture diluted to 0.1 or 0.2 Y.U./mL from intermediate culture and was continued until the culture reached a density of 1.5-3 Y.U. *TEF2*-driven yeast- MUC1 expression is constitutive, and growth of these cells was continued until the cultures reached a density of between 1.1 to 4.0 Y.U./ml. The cells from each culture were then harvested, washed and heat-killed at 56°C for 1 hour in PBS.

[00181] After heat-kill of the cultures, the cells were washed three times in PBS. Total protein expression was measured by a TCA precipitation/nitrocellulose binding assay and by Western blot using an anti-his tag monoclonal antibody and an anti-MUC1 (VNTR) antibody (sc-7313, Santa Cruz). Protein content was quantified using semi-quantitative digital imaging methods. GI-6101 was expected to express the MUC1 fusion protein as a membrane associated protein of about 25 kDa.

[00182] Fig. 2A shows expression of the MUC1 antigen in GI-6101 using anti-MUC1 (VNTR) and anti-His antibodies for detection. These results showed good expression of the MUC1 protein. Fig. 2B shows the MUC1 antigen of GI-6101 after deglycosylation with either EdoH or PNGaseF. This figure shows that the GI-6101 fusion protein is expressed as a glycosylated product, since the size of the fusion protein is larger than estimated prior to deglycosylation, but reduces to the expected size (25 kDa) after deglycosylation by EdoH or PNGaseF.

[00183] Quantification of antigen expression in GI-6101 that was grown under standard culture conditions was compared to GI-6101 grown under neutral pH conditions, as described above. The levels of antigen expression were approximately the same using either process (data not shown), demonstrating that the neutral pH process does not alter the level of MUC1 antigen expression by the yeast.

Example 2

[00184] The following example described the production of a yeast-MUC1 immunotherapeutic composition known as GI-6104.

[00185] In this experiment, yeast (*Saccharomyces cerevisiae*) were engineered to express a human MUC1 antigen under the control of the copper-inducible promoter,

CUP1, or the constitutive promoter, *TEF2*, producing yeast-MUC1 immunotherapy compositions. In each case, a fusion protein comprising a MUC1 antigen was produced as a single polypeptide with the following sequence elements fused in frame from N- to C-terminus, represented by SEQ ID NO:18: SEQ ID NO:18 includes the following MUC1 antigens, in the following order from N- to C-terminus: (1) a first MUC1 CD (positions 1-72 of SEQ ID NO:18); (2) a second MUC1 CD (positions 73-144 of SEQ ID NO:18); and (3) a third MUC1 CD (positions 145-216 of SEQ ID NO:18). This fusion protein further included an N-terminal sequence designed to impart resistance to proteasomal degradation and/or stabilize expression (represented in this fusion protein by SEQ ID NO:21). The fusion protein could alternatively be designed using a MUC1 signal sequence (*e.g.,* positions 1-30 of SEQ ID NO:14), a different synthetic N-terminal peptide as described herein, or an N-terminal peptide from a yeast alpha leader sequence such as SEQ ID NO:19 or SEQ ID NO:20. The complete fusion protein including the N-terminal peptide and a hexahistidine C-terminal tag to facilitate identification and/or purification of the protein is a single polypeptide with the following sequence elements fused in frame from N- to C-terminus (represented by SEQ ID NO:17): (1) a synthetic peptide represented by SEQ ID NO:21 (positions 1-6 of SEQ ID NO:17); (2) a first MUC1 CD (positions 7-78 of SEQ ID NO:17); (3) a second MUC1 CD (positions 79-150 of SEQ ID NO:17); (4) a third MUC1 CD (positions 151-222 of SEQ ID NO:17); and (5) a hexahistidine tag (positions 223-228 of SEQ ID NO:17). SEQ ID NO:17 is encoded by the nucleotide sequence represented by SEQ ID NO:16 (codon optimized for yeast expression).

[00186] Briefly, DNA encoding the MUC1 antigens were synthesized, and then inserted at *EcoRI* and *NotI* cloning sites behind the *CUP1* promoter (vector pGI-100) or the *TEF2* promoter (vectors plu011) in yeast 2 μm expression vectors. Nucleotide sequences encoding a C-terminal hexahistidine peptide was added to the plasmid vector to encode the complete fusion protein represented by SEQ ID NO:17. The resulting plasmids were transformed into DH5α for plasmid storage, and into *Saccharomyces cerevisiae* W303α for production of the yeast-MUC1 immunotherapeutic compositions.

[00187] Transformation into *Saccharomyces cerevisiae* was performed by lithium acetate/polyethylene glycol transfection, and primary transfectants were selected on solid minimal plates lacking Uracil (UDM; uridine dropout medium). Colonies were selected by growing in U2 (uridine dropout medium) or UL2 (uridine and leucine dropout medium) medium at 30° C.

[00188] The yeast-MUC1 immunotherapy composition comprising a polynucleotide encoding the human MUC1 fusion protein represented by SEQ ID NO:17 under the control of the *CUP1* promoter is also referred to herein as GI-6104.

[00189] Liquid cultures lacking uridine (U2) or lacking uridine and leucine (UL2) (media recipes are provided in Example 1) were inoculated using the plates and starter cultures described above, and were grown for about 24h at 30°C, 250 rpm. pH buffered UL2 medium containing 4.2g/L of Bis-Tris (BT-UL2) was also inoculated from frozen yeast banks to evaluate yeast-MUC1 immunotherapeutics produced under neutral pH manufacturing conditions (data not shown). Culturing in pH buffered UL2 medium exposes β-glucans on the yeast cell wall and is believed to modify the cellular immune responses induced by the yeast as a result of modifying the interactions with dectin receptors on antigen presenting cells. The remaining culture conditions were the same whether UL2 or BT-UL2 was used. Primary cultures were used to inoculate final cultures of the same formulation.

[00190] In initial experiments comparing yeast-MUC1 immunotherapeutic compositions under the control of different promoters, *CUP1*-driven (inducible expression) yeast-MUC1 expression was produced in a 2-step or 3-step culture; after starter or intermediate culture reaches mid-log (1.5-4 YU/ml), antigen expression was initiated by the addition of 0.375 mM copper sulfate to final the culture diluted to 0.1 or 0.2 YU/mL from intermediate culture, and was continued until the culture reached a density of 1.5-3 Y.U. *CUP1*-driven (inducible expression) yeast-MUC1 expression was also initiated by the addition of 0.375 mM copper sulfate after the final yeast- MUC1 culture reached a density of approximately 2 Y.U./ml, and was induced for 4-6 hours. *TEF2*-driven yeast- MUC1 expression is constitutive, and growth of these cells was continued until the cultures reached a density of between 1.1 to 4.0 Y.U./ml. The cells from each culture were then harvested, washed and heat-killed at 56°C for 1 hour in PBS.

[00191] After heat-kill of the cultures, the cells were washed three times in PBS. Total protein expression was measured by a TCA precipitation/nitrocellulose binding assay and by Western blot using an anti-his tag monoclonal antibody and an anti-MUC1 (C-terminus) antibody (sc-6827, Santa Cruz). Protein content was quantified using semi-quantitative digital imaging methods. GI-6104 was expected to express the MUC1 fusion protein as a cytosolic protein of about 25 kDa.

[00192] Fig. 2C shows expression of the MUC1 antigen in GI-6104 using anti-MUC1 (C-terminus) and anti-His antibodies for detection. These results showed good expression

of the MUC1 fusion protein. Fig. 2B shows the MUC1 antigen of GI-6104 after deglycosylation with EdoH. This figure shows that the GI-6104 fusion protein is *not* expressed as a glycosylated product, since the size of the fusion protein is the same prior to and after deglycosylation with EdoH.

[00193] Quantification of antigen expression in GI-6104 that was grown under standard culture conditions was compared to GI-6104 grown under neutral pH conditions, as described above. The levels of antigen expression were approximately the same using either process (data not shown), demonstrating that the neutral pH process does not alter the level of MUC1 antigen expression by the yeast.

Example 3

[00194] The following example describes the production of a Yeast-MUC1 agonist immunotherapeutic composition known as GI-6105.

[00195] In this experiment, yeast (*Saccharomyces cerevisiae*) were engineered to express a human MUC1 agonist antigen under the control of the copper-inducible promoter, *CUP1*, producing a yeast-MUC1 agonist immunotherapy composition. The MUC1 agonist antigen was designed using the antigen from the yeast-MUC1 immunotherapy composition of GI-6101 (see Example 1) as a template. Briefly, a fusion protein comprising a MUC1 agonist antigen was produced as a single polypeptide with the following sequence elements fused in frame from N- to C-terminus, represented by SEQ ID NO:23: (1) MUC1 signal sequence (positions 1-30 of SEQ ID NO:23); (2) a MUC1 SEA/ED segment (positions 31-89 of SEQ ID NO:23); (3) a VNTR segment comprising two VNTR domains (positions 90-130 of SEQ ID NO:23); (4) a MUC1 TM domain (positions 131-158 of SEQ ID NO:23); (5) a MUC1 CD (positions 159-230 of SEQ ID NO:23); (6) a MUC1 agonist epitope (positions 231-246 of SEQ ID NO:23) and (7) a hexapeptide histidine tag (positions 247-252 of SEQ ID NO:23). SEQ ID NO:23 is encoded by the nucleotide sequence represented by SEQ ID NO:22 (codon optimized for yeast expression). The MUC1 signal sequence (positions 1-30 of SEQ ID NO:23) could be substituted with a different N-terminal sequence designed to impart resistance to proteasomal degradation and/or stabilize expression, such as the peptide represented by SEQ ID NO:21, or an N-terminal peptide from a yeast alpha leader sequence such as SEQ ID NO:19 or SEQ ID NO:20. hexahistidine C-terminal tag is optional, and facilitates identification and/or purification of the protein. As compared to the antigen in GI-6101 (*e.g.,* SEQ ID NO:14 or 15), the sequence in GI-6105 contains the following amino acid substitutions to create a variety of agonist epitopes (substitution positions given with

reference to SEQ ID NO:23, with further reference to the location of the substitution in a wild-type MUC1 represented by Accession No. NP_001191214): A96Y (position 161 in wild-type MUC1), P97L (position 162 in wild-type MUC1), G104V (position 169 in wild-type MUC1), S105Y (position 170 in wild-type MUC1), T106L (position 171 in wild-type MUC1), A147Y (position 392 in wild-type MUC1), C161V (position 406 in wild-type MUC1), T199L (position 444 in wild-type MUC1), D200F (position 445 in wild-type MUC1), S215Y (position 460 in wild-type MUC1), and T239L (position 93 in wild-type MUC1).

[00196] A plasmid containing the MUC1 agonist antigen for GI-6105 (SEQ ID NO:23) was transfected into W303α yeast and transformants were selected after 3 days of growth at 30°C on uridine dropout agar (UDA). Single colonies were re-streaked onto uridine and leucine dropout agar (ULDA) plates and incubated at 30°C for an additional 4 days to select for cells with elevated plasmid copy number.

[00197] A single colony of GI-6105 was removed from the ULDA plate and used to inoculate 25 mL of UL2 liquid medium (starter culture). The starter culture was incubated with shaking at 30°C to a density of 3.7 YU/mL, and then used to inoculate an intermediate culture to 0.3 YU/mL. The intermediate culture and grown to a density of 3.0 YU/mL, and then used to inoculate a final culture to a density of 0.04 YU/mL. The final culture was grown to a density of 3.6 YU/mL, then treated with 0.5 mM copper sulfate for 3h at 30°C to induce Muc1 agonist v1.0 antigen expression.

[00198] The induced cells were washed once with PBS, heat killed at 56°C for 1h, then thrice washed in PBS. Total protein content of the heat killed cells was measured by Amidoschwarz assay and antigen content was measured by western blot, with a monoclonal antibody recognizing the C-terminal hexahistidine epitope tag. Antigen quantity was determined by interpolation against a standard curve comprised of his tagged HCV NS3 protein. As shown in Fig. 3, the antigen was expressed by the yeast, and the antigen content for GI-6105 was estimated to be approximately 2801 Ng/YU.

Example 4

[00199] The following example describes the production of a Yeast-MUC1 agonist immunotherapeutic composition known as GI-6106.

[00200] In this experiment, yeast (*Saccharomyces cerevisiae*) were engineered to express a human MUC1 agonist antigen under the control of the copper-inducible promoter, *CUP1*, producing a yeast-MUC1 agonist immunotherapy composition. The MUC1 agonist antigen was designed using a full-length wild-type MUC1 antigen having

Accession No. NP_001191214, although other wild-type MUC1 proteins could be utilized to design similar agonists. Briefly, a fusion protein comprising a MUC1 agonist antigen was produced as a single polypeptide with the following sequence elements fused in frame from N- to C-terminus, represented by SEQ ID NO:25: (1) an alpha factor leader sequence disclosed elsewhere herein by SEQ ID NO:19 (positions 1-89 of SEQ ID NO:25); (2) a linker sequence of Thr-Ser (positions 90-91 of SEQ ID NO:25); (3) a full-length MUC1 agonist protein corresponding to a wild-type protein except for the introduction of 11 agonist epitopes (positions 92-566 of SEQ ID NO:25) and (7) a hexapeptide histidine tag (positions 567-572 of SEQ ID NO:25). SEQ ID NO:25 is encoded by the nucleotide sequence represented by SEQ ID NO:24 (codon optimized for yeast expression). The alpha leader sequence (positions 1-89 of SEQ ID NO:25) could be substituted with a different N-terminal sequence designed to impart resistance to proteasomal degradation and/or stabilize expression, such as the peptide represented by SEQ ID NO:21, or an N-terminal peptide from a different yeast alpha leader sequence such as SEQ ID NO:20, or by a MUC1 signal sequence. The hexahistidine C-terminal tag is optional, and facilitates identification and/or purification of the protein. As compared to the wild-type MUC1 protein used as a template, the sequence in GI-6106 contains the following amino acid substitutions to create a variety of agonist epitopes (substitution positions given with reference to SEQ ID NO:25, with further reference to the location of the substitution in a wild-type MUC1 represented by Accession No. NP_001191214): T184L (position 93 in wild-type MUC1), A232Y (position 161 in wild-type MUC1), P233L (position 162 in wild-type MUC1), G240V (position 169 in wild-type MUC1), S241Y (position 170 in wild-type MUC1), T242L (position 171 in wild-type MUC1), A483Y (position 392 in wild-type MUC1), C497V (position 406 in wild-type MUC1), T535L (position 444 in wild-type MUC1), D536F (position 445 in wild-type MUC1), and S551Y (position 460 in wild-type MUC1).

[00201] A plasmid containing MUC1 agonist antigen for GI-6106 was transfected into W303α yeast and transformants were selected after 3 days of growth at 30°C on uridine dropout agar (UDA). Single colonies were re-streaked onto uridine and leucine dropout agar (ULDA) plates and incubated at 30°C for an additional 4 days to select for cells with elevated plasmid copy number.

[00202] A single colony of GI-6106 was removed from the ULDA plate and used to inoculate 25 mL of UL2 liquid medium (starter culture). The starter culture was incubated with shaking at 30°C to a density of ~ 3 YU/mL, and then used to inoculate an

intermediate culture to 0.3 YU/mL. The intermediate culture and grown to a density of 3 YU/mL, and then used to inoculate a final culture to a density of 0.04 YU/mL. The final culture was grown to a density of 3 YU/mL, then treated with 0.5 mM copper sulfate for 3h at 30°C to induce Muc1 agonist v2.0 antigen expression.

[00203] The induced cells were washed once with PBS, heat killed at 56°C for 1h, then thrice washed in PBS. Total protein content of the heat killed cells was measured by Amidoschwarz assay and the agonist antigen content was measured by western blot, with a monoclonal antibody recognizing a C-terminal hexahistidine epitope tag. Antigen quantity was determined by interpolation against a standard curve comprised of his tagged HCV NS3 protein. Results showed that the GI-6106 yeast expressed the antigen (data not shown); antigen content for GI-6106 was estimated to be approximately 2940 Ng/YU.

Example 5

[00204] The following examples describe the phenotypic and functional analysis of the effects yeast-MUC1 immunotherapy compositions on human dendritic cells.

[00205] In order to evaluate the effect of the yeast-MUC1 immunotherapy compositions described in Examples 1 and 2 on the phenotype and function of dendritic cells, the following experiments were performed.

[00206] In a first experiment, human dendritic cells (DCs) were cultured for 48 hours with: (1) media alone (Untreated), (2) CD40L (1μg/ml) plus enhancer for ligands (1 μg/ml) as a positive control; (3) control yeast (Control Yeast; yeast comprising an empty vector (no MUC1 antigen insert)); (4) the yeast-MUC1 immunotherapy composition known as GI-6101, grown under standard growth conditions as described in Example 1 (GI-6101); (5) the yeast-MUC1 immunotherapy composition known as GI-6101, grown under neutral pH growth conditions as described in Example 1 (GI-6101 (DEC)); (6) the yeast-MUC1 immunotherapy composition known as GI-6104 (GI-6104), grown under standard growth conditions as described in Example 2; or (7) the yeast-MUC1 immunotherapy composition known as GI-6104, grown under neutral pH growth conditions as described in Example 2 (GI-6104 (DEC)). Dendritic cells and yeast were combined at a ratio of 1:10 (DC:yeast). DCs were harvested and analyzed by flow cytometry for DC surface-marker expression. The results are shown in Table 1 below as the percentage of positive cells and MFI (parentheses).

Table 1

Treatment of DCs	CD80	CD83	CD86	CD54	Class I	Class II
Untreated	6.4	28.6	97.6	96.3	99.2	80.9
	(3088)	(3352)	(19731)	(14463)	(19582)	(8678)
CD40L	59.3	79.6	99.8	99.6	99.9	81.6
	(3932)	(4364)	(33554)	(44958)	(25589)	(5908)
Control Yeast	41.2	54.7	99.1	96.0	99.3	93.7
	(4479)	(3953)	(49674)	(26634)	(40996)	(8953)
GI-6101	56.2	72.9	99.0	96.0	99.6	83.1
	(5456)	(4654)	(59333)	(41385)	(43290)	(7684)
GI-6101 (DEC)	65.3	74.3	99.6	98.2	99.9	87.0
	(6090)	(4149)	(63934)	(41984)	(33384)	6304)
GI-6104	57.0	65.8	99.0	96.1	99.5	90.6
	(5433)	(4498)	(59460)	(40096)	(42884)	(6992)
GI-6104 (DEC)	52.0	63.0	99.2	97.6	99.9	88.7
	(5502)	(4012)	(55133)	(31823)	(35846)	(6423)

[00207] The results show that yeast (control yeast and yeast expressing MUC1 antigens), regardless of the method of growth, upregulated the expression of CD80 and CD83 on dendritic cells as compared to untreated cells. CD80, or B7.1, is a costimulatory molecule necessary for T cell activation and survival that is upregulated on activated dendritic cells. CD83 is a marker of dendritic cell maturation. Accordingly, this experiment shows that the yeast-MUC1 immunotherapeutic compositions can upregulate DC maturation markers.

[00208] In a second experiment, dendritic cell cytokine and chemokine production were evaluated after culture with the yeast-MUC1 immunotherapeutics. Briefly, human DCs from a normal donor (a donor who was believed to be cancer-free) were cultured for 5 days with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), or treated with CD40L (1 μg/ml, Enzo Life Sciences) plus enhancer for ligands (1 μg/ml, Enzo Life Sciences) for 24 hours, or with Control Yeast (empty vector), GI-6101 cultured under standard growth conditions (GI-6101), GI-6101 cultured under neutral pH conditions (GI-6101 (DEC)), GI-6104 cultured under standard growth conditions (GI-6104), or GI-6104 cultured under neutral pH conditions (GI-6104 (DEC)) for 48 hrs (DC:yeast ratio = 1:10). Cultured supernatants were collected and screened for cytokine and chemokine production by multiplex cytokine/chemokine kit. Results are expressed in pg/ml, and shown in Table 2.

Table 2

Treatment of DCs	IL-2	IL-8	IL-12p70	IL-1β	GM-CSF	IFN-γ	IL-6	IL-10	TNF-α
CD40L	14.9	829.6	152.8	6.3	5069.8	22.3	933.2	74.3	8103.2
Control Yeast	2.8	129.4	316.1	1.8	4420.3	6.3	49.2	3.9	333.7
GI-6101	3.5	183.3	443.3	2.1	4174.8	5.9	75.3	4.4	487.1
GI-6101 (DEC)	**11.5**	**866.6**	**864.2**	4.3	3697.8	**31.8**	**192.0**	10.7	**2148.2**
GI-6104	4.5	315.7	568.0	1.9	4156.0	7.3	121.8	5.0	477.0
GI-6104 (DEC)	**6.5**	**725.2**	**587.8**	3.2	3165.5	**13.2**	**140.4**	8.5	**642.0**

[00209] The results in Table 2 shown that the treatment of dendritic cells from a normal donor with yeast-MUC1 immunotherapy compositions described in Examples 1 and 2 increases cytokine and chemokine production by these cells. Notably, interferon-γ (IFN-γ) production was increased after exposure to yeast-MUC1 immunotherapy compositions grown under neutral pH conditions, which is expected to enhance TH1 and CD8+ T cell responses. In addition, yeast-MUC1 immunotherapy compositions grown under neutral pH conditions induce higher cytokine and chemokine production by DCs, with the yeast-MUC1 immunotherapy composition known as GI-6101 (neutral pH) showing the highest stimulation of DC cytokine and chemokine production. Numbers highlighted in bold type show cytokine/chemokine induction that is statistically significantly improved as compared to untreated control (data not shown).

[00210] The experiment shown in Table 2 was repeated using dendritic cells isolated from a different normal donor. The results (data not shown) are comparable to those shown in Table 2 and confirm that yeast-MUC1 immunotherapy compositions induce cytokine and chemokine production by dendritic cells, and that the yeast-MUC1 composition known as GI-6101, grown under neutral pH growth conditions, induces the highest levels of cytokine and chemokine production by dendritic cells among the two yeast-MUC1 compositions grown under each condition.

[00211] Taken together, these results show that yeast-MUC1 immunotherapy compositions can activate dendritic cells and induce cytokine and chemokine production that is associated with a productive immune response.

Example 6

[00212] The following example shows that yeast-MUC1 immunotherapy compositions of the invention can activate MUC1-specific T cells.

[00213] T-3-P93L is a MUC-1 specific T cell line that specifically recognizes the MUC1 agonist peptide, denoted P93L, in the context of HLA-A2. P93L is a peptide spanning positions 92-101 of a full-length MUC1 protein (*e.g.,* ATWGQDVTSV; positions 92-101 of SEQ ID NO:11), except that the threonine at position 2 of this peptide (position 93 in positions 92-101 of SEQ ID NO:11) is substituted with a leucine, creating an agonist peptide. P93L binds to HLA-A2 at higher levels than the native (wild-type) peptide, and is a better inducer of MUC1-specific T cells than the native peptide (higher production of TH1 cytokines (see U.S. Patent Application Publication No. 2008/0063653). The T cell line T-3-P93L can specifically lyse HLA-A2-positive, MUC1-positive tumor targets *in vitro* (data not shown). This T cell line is specific for a portion of MUC1 that is within the MUC1-N subunit.

[00214] In this experiment, DCs from a normal donor, prepared as described in Example 5 were treated with the yeast-immunotherapy compositions described in Examples 1 and 2, or with control yeast (empty vector), CD40L (positive control) or untreated (negative control) using conditions described above in Example 5. DCs treated with control yeast or with CD40L were pulsed with or without the P93 L peptide (P93L was used at 10 μg/ml). Treated DCs were then used as antigen presenting cells (APCs) to evaluate their ability to stimulate the MUC1-specific T cell line T-3-P93L (T cell:DC ratio = 10:1). 24 hour culture supernatants were collected and screened for the secretion of interferon-γ (IFN-γ). The results are shown in Table 3, expressed as the amount of IFN-γ produced by the T cells in pg/ml.

Table 3

DCs	Treatment	MUC-1 peptide	MUC-1-specific T cells	IFN-γ
-	**None**	-	+	<15.6
+	**CD40L**	-	+	<15.6
+	**CD40L**	+	+	**217.1**
+	**Control yeast**	-	+	<15.6
+	**Control Yeast**	+	+	**339.2**
+	**GI-6101**	-	+	**342.1**
+	**GI-6101 (DEC)**	-	+	**393.5**
+	**GI-6104**	-	+	44.2
+	**GI-6104 (DEC)**	-	+	32.3

[00215] The results show that dendritic cells treated with GI-6101, produced under both standard and neutral pH conditions, and which expresses VNTR domains of the MUC1-N subunit, was able to stimulate the MUC1-N-specific T cells to produce

significant amounts of IFN-γ. GI-6104, which does not express antigen from the MUC1-N protein (GI-6104 only expresses MUC1 antigen from the cytoplasmic domain (CD)), did not stimulate the MUC1-N-specific T cells.

[00216] In a second experiment, the MUC1-C-specific T cell line, denoted T-15-P1240(1Y), was stimulated with DCs that had been treated as in the experiment above, to determine whether yeast-MUC1 immunotherapy compositions of the invention could stimulate these T cells. The T-15-P1240(1Y) cell line is a MUC-1 specific T cell line that specifically recognizes the MUC1 agonist peptide, denoted P1240(1Y), which is a MUC1-C peptide, in the context of HLA-A2. P1240(1Y) is a peptide spanning positions 1240-1248 of a full-length MUC1 protein (*e.g.,* SLSYTNPAV; positions 1240-1248 of SEQ ID NO:11), except that the serine at position 1 of this peptide (position 1240 in positions 1240-1248 of SEQ ID NO:11) is substituted with a lysine, creating an agonist peptide. P1240(1Y) binds to HLA-A2 as well or better than the native (wild-type) peptide, and is a better inducer of MUC1-specific T cells than the native peptide (higher production of TH1 cytokines. The T cell line T-15-P1240(1Y) can specifically lyse HLA-A2-positive, MUC1-positive tumor targets *in vitro* (data not shown). This T cell line is specific for a portion of MUC1 that is within the MUC1-C subunit, and specifically, the cytoplasmic domain (CD).

[00217] In this experiment, DCs were generated from PBMCs of a healthy HLA-A2 positive donor, and prepared as described in Example 5. The DCs were treated with the yeast-immunotherapy compositions described in Examples 1 and 2, or with CD40L (positive control) or untreated (negative control) using conditions described above in Example 5. DCs treated with CD40L were pulsed with or without the P1240(1Y) peptide (peptide was used at 10 μg/ml). Treated DCs were then used as antigen presenting cells (APCs) to evaluate their ability to stimulate the MUC1-specific T cell line T-15-P1240(1Y) (T cell:DC ratio = 10:1). 24 hour culture supernatants were collected and screened for the secretion of interferon-γ (IFN-γ). The results are shown in Table 4, expressed as the amount of IFN-γ produced by the T cells in pg/ml.

Table 4

DCs	Treatment	MUC1-C peptide	MUC1-C-specific T cells	IFN-γ
-	-	-	+	106.1
+	**CD40L**	-	+	<15.6
+	**CD40L**	+	+	**2402.8**
+	**GI-6101**	-	+	**852.1**
+	**GI-6101**	-	-	67.4
+	**GI-6101/ DEC**	-	+	**1667.9**
+	**GI-6101/ DEC**	-	-	113.7
+	**GI-6104**	-	+	**583.6**
+	**GI-6104**	-	-	103.4
+	**GI-6104/DEC**	-	+	**1155.1**
+	**GI-6104/DEC**	-	-	63.6

[00218] The results show that both GI-6101 and GI-6104, grown under standard or neutral pH conditions, were able to stimulate the MUC1-C-specific T cells to produce significant amounts of IFN-γ. Yeast-MUC1 immunotherapy compositions grown under neutral pH conditions (both GI-6101 and GI-6104) stimulated higher levels of IFN-γ production by the T cells than yeast-MUC1 immunotherapy compositions grown under standard conditions.

[00219] In a third experiment, the experiment described in Table 4 above was repeated, but using different DC:T cell ratios for DCs treated with the yeast-MUC1 immunotherapy compositions GI-6101 and GI-6104. The results are presented below in Table 5.

Table 5

DCs	Treatment	MUC1-C peptide	DC:T cell	MUC1-C-specific T cells	IFN-γ (pg/ml)
-	**GI-6101**	-		+	48
+	**GI-6101**	-		-	<15.6
+	**GI-6101/ DEC**	-		-	<15.6
+	**GI-6104**	-		-	<15.6
+	**GI-6104/DEC**	-		-	<15.6
+	**GI-6101**	-	10:1	+	**981.8**
			20:1	+	**628.4**
			40:1	+	**426.4**
+	**GI-6101/DEC**	-	10:1	+	**2039.1**
			20:1	+	**1074.3**
			40:1	+	**768.4**
+	**GI-6104**	-	10:1	+	**534.2**
			20:1	+	**514.6**
			40:1	+	**330.9**
+	**GI-6104 /DEC**	-	10:1	+	**1177.8**
			20:1	+	**824.4**
			40:1	+	**674.6**
+	**CD40 L**	+		+	**2275.4**

[00220] The results again show that both GI-6101 and GI-6104, grown under standard or neutral pH conditions, were able to stimulate the MUC1-C-specific T cells to produce significant amounts of IFN-γ, and that the compositions grown under neutral pH conditions stimulated higher levels of IFN-γ production by the T cells than yeast-MUC1 immunotherapy compositions grown under standard conditions. The results further show a dose response as the number of DCs increases relative to the number of T cells.

[00221] Taken together, these results show that yeast-MUC1 immunotherapy compositions can activate MUC1-specific T cells in an antigen-specific manner, as illustrated by the IFN-γ release from T cells stimulated by DCs treated with the yeast-MUC1 immunotherapy compositions. These results also show an advantage for the production of IFN-γ by T cells as a result of using yeast-MUC1 immunotherapy compositions grown under neutral pH conditions.

Example 7

[00222] The following example demonstrates that yeast-MUC1 compositions of the invention can expand and stimulate MUC1-specific T cells from cancer patients.

[00223] In this experiment, DCs are prepared from the PBMCs of cancer patients (post treatment with a cancer therapy, which can include chemotherapy or viral vaccine

treatment, and/or pre-treatment). The DCs are prepared in a 5-day culture in presence of GM-CSF and IL-4, followed by incubation in presence of yeast (GI-6101 and/or GI-6104, cultured under standard or neutral pH conditions). After 48-hours co-culture, the DCs are used as APCs for stimulation of autologous T cells by measuring cytokine production and/or proliferation of CD4+ T cells. Yeast-MUC1 immunotherapy compositions are expected to expand and activate T cells from the cancer patients.

[00224] In a second experiment, DCs are prepared from the PBMCs of cancer patients (post treatment with a cancer therapy, which can include chemotherapy or viral vaccine treatment, and/or pre-treatment). The DCs are prepared in a 5-day culture in presence of GM-CSF and IL-4, followed by incubation in presence of yeast (GI-6101 and/or GI-6104, cultured under standard or neutral pH conditions). After 48-hours co-culture, the DCs are used as APCs for stimulation of autologous T cells. Each cycle of IVS consists of 3 days in absence of IL-2, following by 4 additional days in presence of 20 U/ml of recombinant IL-2. Tetramers specific for a MUC1 peptide are used to detect the percentage of CD8+ T cells that are expanded by the treatment with the yeast-MUC1 immunotherapy compositions. Yeast-MUC1 immunotherapy compositions are expected to expand and activate T cells from the cancer patients.

[00225] In a third experiment, yeast-MUC1 immunotherapeutic compositions are used to generate MUC1-specific CTLs from PBMCs that lyse MUC1-expressing targets. In this experiment, MUC1-specific T cells from normal donors and/or from cancer patients (post treatment with a cancer therapy, which can include chemotherapy or viral vaccine treatment, and/or pre-treatment), are expanded *in vitro* using DCs incubated with yeast-MUC1 immunotherapy compositions (GI-6101 or GI-6104) for 2 cycles of *in vitro* stimulation (IVS). At day 5, $CD8^+$ T cells are isolated and used in an overnight CTL assay against tumor cells that express MUC1. These experiments are expected to demonstrate that yeast-MUC1 immunotherapeutic compositions can generate MUC1-specific CTLs that are capable of killing a MUC1-expressing tumor cells.

Example 8

[00226] The following example demonstrates that immunization with a yeast-MUC1 immunotherapeutic composition reduces MUC1-expressing tumors *in vivo*.

[00227] In this experiment, mice receive tumor cells expressing a recombinant human MUC1 protein via the tail vein (day 0). Four days post-tumor implantation, animals receive weekly vaccinations with yeast control (YVEC, or empty vector yeast) versus yeast-MUC1 (GI-6101 or GI-6104), administered at a dose of 1YU per site at four

different sites (4YU total per dose). At day 40 post-tumor implantation, animals are sacrificed and the number of lung tumor nodules are evaluated. It is expected that the yeast-MUC1 immunotherapy compositions are capable of reducing MUC1-expressing tumors in mice, as compared to mice receiving yeast alone (no MUC1 antigen).

Example 9

[00228] The following example describes a phase 1 clinical trial in subjects with MUC1-positive cancer.

[00229] An open-label, dose-escalation phase 1 clinical trial is run using a yeast-MUC1 immunotherapy composition known as GI-6101 described in Example 1 or GI-6104 described in Example 2 (grown either under standard growth conditions or under neutral pH conditions). 12-24 subjects with a MUC1-positive tumor are administered the yeast-MUC1 immunotherapy composition in a sequential dose cohort escalation protocol utilizing dose ranges of 4 Y.U. (1 Y.U. x 4 sites), 16 Y.U. (4 Y.U x 4 sites) and 40 Y.U. (10 Y.U. x 4 sites), administered subcutaneously. The yeast-MUC1 immunotherapy is administered at 2 week intervals for 3 months, and then monthly. An expansion cohort of patients (n=10) at maximum tolerated dose (MTD) or the observed best dose are selected for additional study. The results monitor safety as a primary endpoint, and as secondary endpoints, antigen-specific T cell responses (*e.g.,* MUC1-specific $CD8^+$ T cells emerging or expanding on treatment) as well as clinical activity.

[00230] GI-6101 and GI-6104 are expected to be safe and well-tolerated with no significant toxicities. In addition, GI-6101 or GI-6104 are expected to produce treatment-emergent MUC1-specific T cell responses or an improvement in pre-existing MUC1-specific baseline T cell responses in a statistically significant number of patients. Some patients are also expected to have stabilized disease.

Example 10

[00231] The following example describes a clinical trial (P1/P2) using Yeast-MUC1 immunotherapeutic compositions.

[00232] Increased MUC1 expression has been observed in ~ 70% of the acute myeloid leukemia (AML) cases, suggesting that elevated MUC1 levels may be involved in regulating the proliferative potential of the immature leukemic compartment.

[00233] In a first clinical trial, first-line use of the yeast-based immunotherapy product known as GI-6101 (see Example 1) is implemented in the setting of MUC1-positive AML (this trial design is also applicable to other yeast-MUC1 immunotherapy compositions, such as GI-6104). The use of GI-6101 is designed to complement existing cytotoxic

standard of care regimens, cytarabine and an anthracycline (*e.g.,* daunorubicin), in an add-on approach by promoting immune killing of MUC1-positive leukemic cells, as well as eliminating MUC1 leukemic cells which can escape terminal differentiation (apoptosis) pathways. Endpoints include improvements in the induction of remission as well as overall survival (pre- and post-transplant).

[00234] In a second trial, GI-6101 is used in the bone marrow transplantation (BMT) setting to prevent relapse of AML in patients with MUC1-positive disease (this trial design is also applicable to other yeast-MUC1 immunotherapy compositions, such as GI-6104). Clinical strategies which evaluate the vaccination of bone marrow donors (adoptive transfer) and/or vaccination of bone marrow recipients in the post transplant period are used to reduce the rate of relapse after BMT.

(A) Clinical Study design for first line therapy of MUC1-positive AML patients with GI-6101 plus cytaribine and daunorubicin versus standard of care alone.

[00235] Patients receive induction chemotherapy consisting of continuous intravenous infusion of cytaribine (cytosine arabinoside) at 100-200mg/m2 per day x 7 days plus intravenous daunoribicin (or daunomycin (daunomycin cerubidine)) 45mg/m2 on days 1, 2, and 3 of cytaribine therapy, or an accepted variation of this regimen, followed by GI-6101 (or placebo) administration 14 days after completion of the induction cycle of chemotherapy. GI-6101 (or placebo) are then administered 14 days after re-induction therapy or 14 days after every subsequent consolidation cycle of chemotherapy. After induction, re-induction, and consolidation therapy, GI-6101 (or placebo) are administered each month for up to 3 years with the primary objective of preventing relapse of remission.

[00236] It is expected that the use of GI-6101 enhances the relapse of remission in patients as compared to those receiving placebo.

(B) Clinical Study design for post-BMT therapy of MUC1-positive AML with GI-6101 versus placebo.

[00237] For MUC1-positive AML patients who require myelo-ablative therapy followed by bone marrow transplant, GI-6101 (or placebo) are administered to the bone marrow donor 7-14 days prior to the donation of bone marrow, and GI-6101 (or placebo) are administered to the bone marrow recipient on a monthly basis for up to 3 years after bone marrow engraftment occurs. The primary objective is to reduce the rate of AML relapse.

[00238] It is expected that the use of GI-6101 reduces the rate of relapse in AML patients as compared to those patients taking placebo.

[00239] While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following exemplary claims.

What is claimed is:

1. A Yeast-MUC1 immunotherapeutic composition, wherein the immunotherapeutic composition comprises:

a) a yeast vehicle; and

b) at least one MUC1 antigen expressed by the yeast vehicle, wherein the MUC1 antigen comprises an amino acid sequence that is at least 95% identical to SEQ ID NO:25 or to positions 92-566 of SEQ ID NO:25, and wherein the MUC1 antigen comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

2. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the MUC1 antigen comprises an amino acid sequence of SEQ ID NO:25.

3. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the yeast vehicle is a whole yeast.

4. The Yeast-MUC1 immunotherapeutic composition of Claim 3, wherein the whole yeast is heat-inactivated.

5. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the yeast vehicle is from a mutant yeast strain that produces underglycosylated proteins, as compared to a wild-type yeast strain.

6. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the yeast vehicle is from *Saccharomyces cerevisiae*.

7. The Yeast-MUC1 immunotherapeutic composition of claim 1, wherein the MUC1 antigen comprises an amino acid sequence that is at least 95% identical to positions 92-566 of SEQ ID NO:25, and wherein the MUC1 antigen comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

8. The Yeast-MUC1 immunotherapeutic composition of claim 1, wherein the MUC1 antigen comprises an amino acid sequence that is at least 95% identical to SEQ ID NO:25, and wherein the MUC1 antigen comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

9. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the MUC1

antigen comprises an amino acid sequence that is at least 97% identical to positions 92-566 of SEQ ID NO:25, and wherein the MUC1 antigen comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

10. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the MUC1 antigen comprises an amino acid sequence that is at least 97% identical to SEQ ID NO:25, and wherein the MUC1 antigen comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

11. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the MUC1 antigen comprises an amino acid sequence that is at least 99% identical to positions 92-566 of SEQ ID NO:25.

12. The Yeast-MUC1 immunotherapeutic composition of Claim 1, wherein the MUC1 antigen comprises an amino acid sequence that is at least 99% identical to SEQ ID NO:25.

13. A Yeast-MUC1 immunotherapeutic composition, wherein the immunotherapeutic composition comprises:

a) a yeast vehicle; and

b) at least one MUC1 antigen expressed by the yeast vehicle, wherein the MUC1 antigen comprises an amino acid sequence that is at least 95% identical to SEQ ID NO:25 or to positions 92-566 of SEQ ID NO:25, and wherein the MUC1 antigen comprises at least one of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

14. The Yeast-MUC1 immunotherapeutic composition of claim 1, wherein the MUC1 antigen comprises each of the following amino acids L184, Y232, L233, V240, Y241, L242, Y483, V497, L535, F536, and Y551.

15. The Yeast-MUC1 immunotherapeutic composition of claim 1, wherein the MUC1 antigen consists of positions 92-566 of SEQ ID NO:25.

16. The Yeast-MUC1 immunotherapeutic composition of Claim 13, wherein the yeast vehicle is a whole yeast.

17. The Yeast-MUC1 immunotherapeutic composition of Claim 16, wherein the whole yeast is heat-inactivated.

18. The Yeast-MUC1 immunotherapeutic composition of Claim 13, wherein the yeast vehicle is from a mutant yeast strain that produces underglycosylated proteins, as compared to a wild-type yeast strain.

19. The Yeast-MUC1 immunotherapeutic composition of Claim 13, wherein the yeast vehicle is from *Saccharomyces cerevisiae*.

20. A Yeast-MUC1 immunotherapeutic composition, wherein the immunotherapeutic composition comprises:

a) a yeast vehicle; and

b) a MUC1 antigen expressed by the yeast vehicle, wherein the MUC1 antigen comprises an amino acid sequence that is at least 98% identical to SEQ ID NO:25 or to positions 92-566 of SEQ ID NO:25.

21. The Yeast-MUC1 immunotherapeutic composition of Claim 20, wherein the yeast vehicle is a whole yeast.

22. The Yeast-MUC1 immunotherapeutic composition of Claim 21, wherein the whole yeast is heat-inactivated.

23. The Yeast-MUC1 immunotherapeutic composition of Claim 20, wherein the yeast vehicle is from a mutant yeast strain that produces underglycosylated proteins, as compared to a wild-type yeast strain.

24. The Yeast-MUC1 immunotherapeutic composition of Claim 20, wherein the yeast vehicle is from *Saccharomyces cerevisiae*.