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(71) Applicant *(for all designated States except US)*: **THE REGENTS OF THE UNIVERSITY OF COLORADO** [US/US]; 1800 Grant Street, 8th Floor, Denver, CO 80203 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BELLGRAU, Donald**; 1874 Ridgecrest Way, Highlands Ranch, CO 80129 (US). **WAZIRI, Allen** [US/US]; 725 Forest Street, Denver, CO 80220 (US).

(74) Agent: **DOMITROVICH, Angela**; Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, CO 80202 (US).

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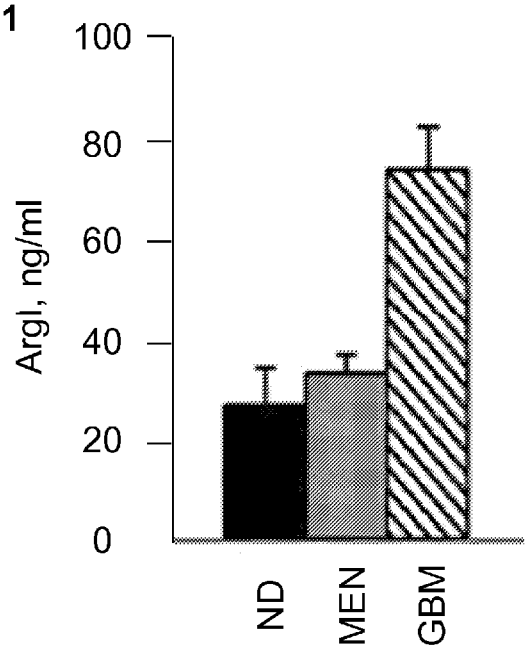
(54) Title: COMBINATION YEAST-BASED IMMUNOTHERAPY AND ARGININE THERAPY FOR THE TREATMENT OF MYELOID-DERIVED SUPRESSOR CELL-ASSOCIATED DISEASES

FIG. 1



(57) Abstract: Disclosed is a method for the prevention and/or treatment of diseases associated with myeloid-derived suppressor cell (MDSC)-mediated immunosuppression and/or arginase-mediated immunosuppression, including without limitation cancer. The method includes the combination of yeast-based immunotherapy and arginine therapy. Compositions and kits encompassed by the invention are also described.

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Combination Yeast-Based Immunotherapy and Arginine Therapy for the Treatment of Myeloid-Derived Suppressor Cell-Associated Diseases

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 61/370,972, filed August 5, 2010. The entire disclosure of U.S. Provisional Patent Application No. 61/370,972 is incorporated herein by reference.

REFERENCE TO A SEQUENCE LISTING

[0002] This application contains a Sequence Listing submitted electronically as a text file by EFS-Web. The text file, named "3923-31-PCT_ST25", has a size in bytes of 56KB, and was recorded on 3 August 2011. 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

[0003] This invention generally relates to the combination of yeast-based immunotherapy and arginine therapy for the treatment of diseases characterized by MDSC-mediated and/or arginase-mediated immunosuppression, including cancer.

BACKGROUND OF THE INVENTION

[0004] Cells of myeloid lineage have been increasingly associated with immunosuppression in a number of systems, including various forms of cancer. Myeloid-derived cells at different states of maturation have been studied as potent inactivators of both CD4+ and CD8+ T cells (Marigo et al., *Immuno Rev* 2008;222:162-79). Populations of immature myeloid cells as well as more mature, differentiated monocytes and granulocytes have been previously shown to possess immunosuppressive abilities (Gabrilovich et al., *Nat Rev Immunol.* 2009;9:162-74; Peranzoni et al., *Curr Opin Immunol* 2010;22:239-44; Serafini et al., *Semin Cancer Biol.* 2006 Feb;16(1):53-65). Given prior observations of T cell dysfunction in patients with glioblastoma multiforme (GBM) and the documentation of myeloid cells with immunosuppressive characteristics in patients with other cancers, efforts have been made to identify a myeloid-derived source of peripheral immunosuppression in GBM patients.

[0005] Immunotherapy has become a focus of recent research in GBM and other cancers due to the potential for combined target specificity and sensitivity. Numerous groups have tested immunotherapeutic strategies in patients with GBM. Unfortunately, as has been seen with similar approaches in most other cancers, these efforts have been in large part unsuccessful (reviewed in Rolle et al., *Neurosurg Clin N Am* 2010; 21:210-14). A major potential pitfall for immunotherapy in GBM is the known suppression of cellular immunity seen in affected patients, which has been well described over the past few decades. Many groups have reported on the variety of functional defects seen in the circulating pool of T cells from these individuals (Waziri, *Neurosurg Clin N Am* 2010;12:31-42). Some investigators have documented the exceedingly rare and ultimately ineffectual T cell infiltrates found within GBM (Waziri et al., *J Immunol* 2008;180:7673-80). In spite of these apparently local as well as global aberrations in cellular immunity, patients with GBM are not systemically immunocompromised. This fact, combined with the potential for recovery of cellular immune function following surgical resection (Brooks et al., *J Neurosurg* 1981; 54:331-7), has implicated a tumor-derived factor in the suppression of cell-mediated immune responses. It is therefore likely that tumor-associated immunosuppressive factors will similarly affect clinical attempts to augment anti-tumor responses. Therefore, targeting tumor-associated immunosuppression in patients with GBM and other cancers is believed to be an avenue for the development of meaningful immunotherapeutic strategies.

[0006] A mechanism common to MDSC-related immunosuppression in most systems is the expression of the enzyme Arginase I (ArgI), which catalyzes the depletion of arginine (Arg). ArgI has been shown to exert immunosuppressive effects through the consumption of L-arginine. L-arginine is a critical cofactor for sustained T cell activation due to a central role in the re-expression of the T cell co-receptor CD3ζ (Rodriguez et al., *J Immunol* 2003;171:1232-9). When released into the extracellular environment, ArgI can potently and rapidly deplete extracellular L-arginine, resulting in T cell anergy and immune dysfunction. Neutrophil degranulation and subsequent release of ArgI have previously been linked to immunosuppression in renal cell carcinoma (Zea et al., *Cancer Res* 2005;65:3044-8; Rodriguez et al., *Cancer Res* 2009;69:1553-1560) and non-small cell lung cancer (Rotondo et al., *Int J Cancer 2009*;125:887-93). MDSCs have also been implicated as immunosuppressors in breast cancer, pancreas cancer and colorectal cancer.

[0007] L-arginine supplementation has been previously utilized within non-cancer clinical settings. On an initially empiric basis, oral arginine supplementation was explored and found to demonstrate efficacy for improving immune function in patients suffering major trauma or undergoing extensive surgical procedures (Neilly et al., *Ulster Med J* 1994;64:193-200). It was subsequently confirmed that ArgI is transiently found at increased levels in these patients (Ochoa et al., *Ann Surg* 2009;3:393-9; Tsuei et al., *J Trauma* 2001;51:497-502), supporting the clinical utility for dietary L-arginine supplementation in the reversal of immunosuppression. However, prior to the present invention and to the inventors knowledge, oral L-arginine supplementation has not been used as a therapy, or demonstrated to be effective to reverse MDSC-derived immunosuppression, in cancer patients.

[0008] 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.

[0009] Treatment of patients diagnosed with glioblastoma multiforme (GBM), as well as several other cancers, is one of the lasting challenges of modern medicine. In GBM, for example, responses to standard external beam radiotherapy and chemotherapy remain dismal and provide for limited improvement in survival. Most experimental therapies have proven ineffective and the overall clinical strategy for affected individuals has not changed significantly in basic design for several decades. In spite of all best attempts, tumor recurrence is a nearly uniform phenomenon and the significant majority of patients succumb to progressive brain disease in just over one year from diagnosis (Wen et al., *N Engl J Med* 2008;359:492-507). New approaches for developing effective and targeted treatment options are needed for patients affected with GBM, and with a variety of other cancers that have proven to be difficult to treat using conventional approaches.

SUMMARY OF THE INVENTION

[0010] One embodiment of the invention relates to a method to treat a disease associated with myeloid-derived suppressor cell (MDSC)-mediated or arginase-mediated immunosuppression. The method includes the step of administering to an individual with the disease a yeast-based immunotherapy composition and arginine therapy.

[0011] In one aspect, the arginine therapy comprises administering an oral arginine supplement to the individual. In one aspect, the arginine supplement is L-arginine. In one aspect, the L-arginine is administered daily. In one aspect, the arginine supplement is administered as a powder, a tablet, or a capsule. In one aspect, the arginine therapy comprises administration of an arginase inhibitor to the individual. In one aspect, the arginase inhibitor is selected from the group consisting of: nor-NOHA (N(omega)-Hydroxy-nor-L-arginine) and NOHA (NG-Hydroxy-L-arginine).

[0012] In one aspect of this embodiment of the invention, the yeast-based immunotherapy composition comprises a yeast vehicle that recombinantly expresses an antigen associated with the disease. In one aspect, the yeast vehicle is a whole, heat-killed yeast. In one aspect, the yeast vehicle is from *Saccharomyces*. In one aspect, the yeast-based immunotherapy composition is administered weekly for 1-5 weeks, followed by monthly administration for 3-24 months.

[0013] In one aspect of this embodiment of the invention, the disease is cancer, including, but not limited to, melanomas, squamous cell carcinoma, breast cancers, head and neck carcinomas, thyroid carcinomas, soft tissue sarcomas, bone sarcomas, testicular cancers, prostatic cancers, ovarian cancers, bladder cancers, skin cancers, brain cancers, angiosarcomas, hemangiosarcomas, mast cell tumors, leukemias, lymphomas, primary hepatic cancers, lung cancers, pancreatic cancers, gastrointestinal cancers (including colorectal cancers), renal cell carcinomas and other kidney cancers, hematopoietic neoplasias or metastatic cancers thereof.

[0014] In one aspect, a cancer antigen is selected from, but is not limited to: carcinoembryonic antigen (CEA) and epitopes thereof such as CAP-1, CAP-1-6D; MART-1; MAGE-1; MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, MAGE-3; GAGE; LAGE-1, GP-100; MUC-1; MUC-2; mutated Ras oncoprotein; normal and mutated p53 oncoproteins; PSMA; tyrosinase; TRP-1 (gp75); NY-ESO-1; NY-BR-1; CTAG1; BAGE-1; GCC; CA9; SCP-1, SSX-2, SSX-4, TRP-2; TAG72; KSA; CA-125; PSA; HER-2/neu/c-erb/B2; EGFR; hTERT; p73; B-RAF; adenomatous polyposis coli (APC); Myc; von Hippel-Lindau protein (VHL); Rb-1; Rb-2; androgen receptor (AR); Smad4; MDR1 (also known as P-glycoprotein); Flt-3; BRCA-1; BRCA-2; Bcr-Abl; pax3-fkhr; ews-fli-1; Brachyury; HERV-H (human endogenous retrovirus H); HERV-K (human endogenous retrovirus K), including but not limited to HERV-K-MEL; TWIST; Mesothelin; NGEP; modifications of such antigens and tissue specific antigens; splice variants of such

antigens; and/or epitope agonists of such antigens. In one aspect, the antigen is an epidermal growth factor receptor (EGFR) antigen.

[0015] In one aspect, antigen is a cancer antigen expressed by a brain cancer, including but not limited to, glioblastoma multiforme. In one aspect, the cancer antigen is selected from: epidermal growth factor receptor (EGFR) including EGFRvIII, mutated Ras (having a mutation at position 12, 13, 59 and/or 61), mitogen-activated protein kinase (MAPK), interleukin-13 receptor-α2 (IL-13Rα2), gp100, TRP-2, MAGE-A3, MAGE-1, Her-2/neu, EphA2, survivin, Wilm's Tumor 1 (WT1), Sry-Related High-Mobility Group Box-2 (SOX2), SOX11, AIM2, and Squamous Cell Carcinoma Antigen Recognized by T Cells 1 (SART1).

[0016] In one aspect, the antigen is a cancer antigen expressed by a lung cancer. In one aspect, the antigen is selected from: mutated Ras (mutations at positions 12, 13, 59 and/or 61), carcinoembryonic antigen (CEA), mucin-1 (MUC1), Brachyury, EGFR, Her2/neu, NY-ESO-1, and MAGE-A3.

[0017] In one aspect, the antigen is a cancer antigen expressed by a renal cell carcinoma. In one aspect, the antigen is selected from: CEA, MUC1, Brachyury, mutated Ras, EGFR, multidrug resistance-associated protein 3, polycomb group protein enhancer of zeste homologue 2, Her2/neu, von Hippel-Lindau (VHL) protein, kidney cancer antigen 1 (KCAG1), TRP-2, FGF-5, and renal cell carcinoma-associated antigen G250.

[0018] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with breast cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, TWIST, hTERT, Her2/neu, NY-BR-1, EphA2, CTAG1, carbonic anhydrase IX (CA9), MAGE-A3, MAGE-1, BAGE-1, HERV-K and HERV-H.

[0019] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with pancreas cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, LAGE-1, NY-ESO-1, SCP-1, SSX-2, SSX-4, HERV-K-MEL and HERV-H.

[0020] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with colorectal cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, CTAG1, MAGE-A3, MAGE-1, BAGE1, GCC, Her2/neu, carbonic anhydrase IX (CA9), HERV-K and HERV-H.

[0021] In one aspect, wherein the method comprises the steps of: (a) administering the arginine therapy as a monotherapy for a period of from 1 to 30 days prior to administering the yeast-based composition; (b) after step (a), administering the yeast-based composition weekly for five weeks followed by 3 to 24 monthly doses, while continuing to administer the arginine therapy daily; and (c) After step (b), administering the arginine therapy as a monotherapy for an additional 1 to 12 months or longer.

[0022] Another embodiment of the invention relates to a kit comprising a yeast-based immunotherapy composition and an arginine supplement or an inhibitor of arginase. In one aspect, the arginine supplement is L-arginine. In one aspect, the yeast-based immunotherapy composition comprises a yeast vehicle that recombinantly expresses an antigen associated with the disease. In one aspect, the yeast vehicle is a whole, heat-killed yeast. In one aspect, the antigen is a cancer antigen. In one aspect, the antigen is a cancer antigen from a brain cancer, a lung cancer, a renal cell carcinoma, a breast cancer, a pancreas cancer, and/or a colorectal cancer.

[0023] Yet another embodiment of the invention relates to the use of a yeast-based immunotherapy composition and arginine therapy in the preparation of a medicament for the treatment of a disease.

[0024] Another embodiment of the invention relates to the use of a yeast-based immunotherapy composition and arginine therapy to treat cancer.

BRIEF DESCRIPTION OF THE FIGURES OF THE INVENTION

[0025] Fig. 1 is a graph showing that ELISA analysis of serum confirms significantly elevated levels of ArgI in patients with GBM.

[0026] Fig. 2A is a graph showing that PHA-stimulated peripheral blood mononuclear cells (PBMC) from patients with GBM demonstrate significantly higher levels of arginase I (ArgI) as compared to PBMCs from normal donors (ND) or patients with meningioma (MEN).

[0027] Fig. 2B is a graph showing that sorted myeloid-derived suppressor cells (MDSC) from patients with GBM used in a mixed lymphocyte reaction (MLR) demonstrate significantly higher levels of arginase I (ArgI) as compared to MDSCs from normal donors (ND) or patients with meningioma (MEN).

[0028] Fig. 2C is a graph showing that interferon-γ (IFNγ) production by PBMCs is significantly limited in the presence of MDSCs from GBM patients in PHA-stimulated cultures, as compared to MDSCs from normal donors (ND), MDSCs from patients with

pituitary tumors (PIT), MDSCs or from patients with meningioma (MEN); the addition of arginine (Arg) or arginase inhibitor (NOHA) to the MDSCs from GBM patients reversed the inhibition of IFN-γ production.

[0029] Fig. 2D is a graph repeating the results from Fig. 2C using MLR cultures.

[0030] Fig. 3 is a survival graph showing that a yeast-based immunotherapy composition expressing human EGFR protects against challenge with intracranial tumors expressing human EGFR when administered subcutaneously and intranasally.

[0031] Fig. 4 is a survival graph showing that a yeast-based immunotherapy composition expressing rat EGFR or human EGFR protects against challenge with intracranial tumors expressing rat EGFR.

[0032] Fig. 5A is a survival graph showing that a yeast-based immunotherapy composition expressing human EGFR protects against challenge with intracranial tumors expressing human EGFR.

[0033] Fig. 5B is a graph showing that effector cells from a rat immunized with a yeast-based immunotherapy composition expressing human EGFR can lyse targets expressing human EGFR.

[0034] Fig. 6 is a schematic drawing showing a phase 0 clinical trial design for arginine therapy in GBM patients.

DETAILED DESCRIPTION OF THE INVENTION

[0035] The present invention generally relates to improved methods for treating diseases that are characterized by myeloid-derived suppressor cell (MDSC)-mediated or arginase-mediated immunosuppression, such as cancer. The method includes treating an individual who has or is at risk of having such a disease an immunotherapy composition and arginine therapy, which can include arginine supplementation and/or arginase inhibition. In one aspect, the invention provides for the administration of yeast-based immunotherapy in conjunction with a protocol of arginine therapy (*e.g.,* arginine supplementation and/or inhibition of arginase) in an individual who has cancer associated with MDSC-mediated immunosuppression. Administration of arginine therapy prior to, during, and/or subsequent to immunotherapy such as yeast-based immunotherapy, provides improved cellular immune function and enhances the efficacy of the immunotherapy for treating disease, including cancer, and particularly cancers that are associated with immune suppression associated with MDSC and/or arginase, such as arginase I (ArgI).

[0036] More particularly, one of the present inventors and colleagues have recently identified an expanded population of cells within the peripheral blood of glioblastoma multiforme (GBM) patients that express a pattern of markers previously defined as characteristic of MDSC (Sippel, Waziri, et al., *submitted for publication*). These cells, phenotypically defined as CD11b+/CD33lo/HLA-DRneg/CD14neg, were not found at increased frequency in patients with non-malignant brain tumors. Additional studies confirmed that MDSC within GBM patients express the granulocytic markers CD15 and CD66. To explore potential *in vivo* activity of MDSC, serum samples from patients with GBM were evaluated and found to bear nearly 3-fold higher levels of ArgI than were seen in sera from normal donors or patients with other intracranial tumors. These studies showed that targeting ArgI may provide benefit for restoring cellular immune function in GBM patients, and further confirmed that: 1) ArgI exerts a central and reversible role in the suppression of cellular immune function in patients with GBM, and 2) reversal of ArgI-mediated effects through either pharmacological inhibition or addition of exogenous L-Arg can restore GBM T cell function to levels equivalent with normal controls.

[0037] These data led to several key conclusions regarding immunosuppression in patients with GBM: (1) phenotypic and functional abnormalities within circulating T cells confirm that GBM-specific immunosuppressive effects extend beyond the local brain environment, mandating the presence of an intermediary immunosuppressive agent; (2) patients with GBM harbor significantly increased numbers of circulating MDSC that are associated with increased levels of serum ArgI, which is also applicable to other types of cancer; (3) sorted MDSC from patients with GBM can suppress normal donor T cell responses through an ArgI-specific mechanism, providing functional confirmation for suppressive activity of these cells *in vivo*; (4) targeting ArgI-mediated immunosuppression *in vitro* results in complete restoration of GBM T cell functional responses.

[0038] While L-arginine supplementation has been previously utilized within non-cancer clinical settings, no previous clinical trial has specifically focused upon the use of oral arginine supplementation to reverse MDSC-derived immunosuppression in cancer patients. Since augmentation of T cell function by targeting arginase (*e.g.,* ArgI) *in vivo* may not, in isolation, confer significant clinical benefit sufficient to result in tumor clearance, the present inventors describe herein an innovative approach for the treatment of cancer and other diseases characterized by MDSC-mediated immunosuppression: specifically, targeting MDSC-mediated immunosuppression using arginine therapy to

restore cellular immune function and augment, enhance or boost the efficacy of yeast-based immunotherapy. Indeed, the present inventors, without being bound by theory, propose that while targeting MDSC in isolation is an attractive approach for improving cellular immune function in patients with cancer, a combinatorial approach targeting MDSC with concurrent tumor-specific immune stimulation provides for a more robust and lasting immunological response, thereby providing greater therapeutic benefit to the patient.

[0039] As described in U.S. Patent No. 7,465,454, one of the inventors and colleagues described multiple yeast-based immunotherapy compositions for cancer, and in one example, evaluated the efficacy of a yeast-based composition expressing EGFR in a 9L-EGFR model of rat glioma, which is an animal model that has been used to study glioblastoma multiforme. In this experiment, Fischer rats were immunized in the periphery with yeast expressing an EGFR antigen (denoted EGFR-vax or EGFR-tm VAX in the '454 patent). The results showed that a significant subset of animals immunized with the yeast-EGFR immunotherapy composition were provided long-term protection from intracranial tumor challenge and overall survival was improved with the immunotherapy, with just less than half of the animals surviving through the conclusion of the experiment. Additional experiments showing similar anti-tumor efficacy with other yeast-EGFR immunotherapy compositions are described herein (see Examples). With respect to the remaining treated animals that still succumbed to tumor, it can be hypothesized that tumor-associated immunosuppression ultimately contributed to their demise. Vaccine studies have demonstrated that CD11bc+/His48+ MDSC are induced by tumor in the rat 9L glioma model and are specifically responsible for suppressing not only endogenous T cell responses but also anti-tumor immunity generated via vaccine strategies [Barth et al., *J Neurooncol* (2009) 94:299–312; Jia et al., *J Neuroimmunol.* 2010 June; 223(1-2): 20–30). The present invention provides a unique solution to this problem by treating the MDSC-associated immunosuppression in a manner that allows for concurrent immunization with an immunotherapy product that is an effective against tumors, and that will synergize with the interruption of MDSC-associated immunosuppression to provide an improvement in clinical outcomes, including for cancer, improvements in survival, reduction in tumor burden, and inhibition of tumor growth.

[0040] Yeast-based immunotherapy has been shown to induce innate immune responses, as well as adaptive immune responses against the target antigen, including

CD4-dependent TH17 and TH1 T cell responses and antigen-specific $CD8^+$ T cell responses, which include cytotoxic T lymphocyte (CTL) responses, as well as 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-based 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. Furthermore, other than the use of arginine therapy described herein, yeast-based immunotherapy 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 can be used if desired. Moreover, yeast-based immunotherapy can be administered repeatedly without losing efficacy, as may be problematic with other types of immunotherapy.

[0041] Finally, one of the inventors has previously shown that yeast induce both CD4 TH1 and CD4 TH17 T cells, the former subset which is one means to generate effector CD8 T cells, and the latter subset which reduces CD8 T cell frequencies by interfering with IL-12 production while also preventing the generation of regulatory T cells (Treg) (see PCT Publication No. WO 2011/032119). Therefore while TH17 interfere with TH1-dependent CD8 killer T cell generation, they also permit multiple immunizations by yeast-based immunotherapy without Treg neutralization. Interestingly, high arginase levels have also been associated with TH17 activity. Since it further appears that individuals can be broadly classified into TH17 high and low responders, and since the former might benefit by reducing TH17 activity, resulting in increased CD8 activity, the combination of arginine therapy with yeast-based immunotherapy is predicted to benefit individuals who may have overactive TH17 responses to yeast-based immunotherapy by directing their immune response toward a TH1 and CD8+ response.

[0042] While this description and the examples below describe the use of yeast-based immunotherapy and arginine therapy by illustrating the treatment of glioblastoma multiforme (GBM), the invention is not limited to this cancer, or to the treatment of cancer in general. It is believed that the invention is applicable to any disease or condition that is

characterized by MDSC-mediated or ArgI-mediated immunosuppression, and which is further amenable to prevention or treatment using yeast-based immunotherapy.

[0043] One embodiment of the invention relates to a method to treat a disease characterized by MDSC-mediated or arginase-mediated immunosuppression, which includes the steps of administering to an individual with the disease a yeast-based immunotherapy composition and arginine therapy. In one aspect of the invention, the disease is cancer. The invention further includes various compositions, formulations and kits that can be used to perform the methods of the invention. Various aspects of the invention are described in detail below, but the invention is not limited to these examples.

Arginine Therapy

[0044] Reference herein to "arginine therapy" refers generally to any treatment that increases the level or amount of arginine (L-arginine) in an individual, whether by increasing the level of, or preventing the breakdown of, arginine that is naturally synthesized by the body, and/or by providing arginine from an exogenous source. Accordingly, "arginine therapy" includes the use of "supplemental arginine" (*e.g.,* arginine provided through the diet and/or as a dietary or nutritional supplement) as well as the use of agents that inhibit arginases (*e.g.,* arginase I or ArgI, or arginase II or ArgII) or that catalyze the breakdown of arginine (*e.g.,* the catalysis of L-arginase into L-ornithine and urea).

[0045] Reference to the administration of "supplemental arginine" as used herein refers to any form of supplemental arginine or an analog or derivative of arginine, including, but not limited to, L-arginine and derivatives and analogs thereof, foods that contain arginine, and compositions or products that contain arginine or derivatives or analogs thereof. Supplemental arginine, including L-arginine, can be synthesized *de novo* or purified from natural sources, such as plant or animal sources.

[0046] Arginine is a conditionally nonessential amino acid, meaning most of the time it can be manufactured by the human body, and does not need to be obtained directly through the diet. Animal sources of arginine include, but are not limited to, dairy products, meats, gelatin, and seafood. Plant sources of arginine include, but are not limited to, grains, nuts, seeds, chick peas, and soybeans. Arginine can be synthesized from citrulline by the sequential action of argininocsuccinate synthetase and argininosuccinate lyase. Reference to "arginine" generally refers to any form of arginine, including but not limited to, L-Arginine and any analogs, derivatives or other formulations of arginine. L-Arginine

is commercially available in various forms and/or referred to by different names, including but not limited to, 2-Amino-5-guanidinopentanoic Acid, Arg, Arginine, Arginine Ethyl Ester, Arginine Ethyl Ester Dihydrochloride, Arginine Ethyl Ester HCl, Arginine HCl, Arginine Hydrochloride, Di-Arginine Malate, Di-Arginine Orotate, Di-L-Arginine-L-Malate, Dl-Arginine, L-Arginina, L-Arginine Ethyl Ester Dichloride, L-Arginine HCl, L-Arginine Hydrochloride, L-Arginine L-Pyroglutamate, L-Arginine Pyroglutamate, R-Gene 10. Arginine can be administered orally, in powder, tablet, or capsule form, or can be administered by injection, although administration is not limited to these routes or forms. Oral L-arginine supplementation is clinically attractive due to low cost, ease of delivery, and negligible toxicity, and is able to restore T cell functional responses in the environment of ArgI-mediated immunosuppression.

[0047] 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.

[0048] 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.

[0049] 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.

[0050] According to the present invention, inhibition of an arginase is also an effective means of addressing ArgI-mediated immunosuppression and providing arginine therapy to an individual. Arginase is a manganese-containing enzyme and catalyzes the following reaction: arginine + H2O → ornithine + urea. Arginase I (ArgI) functions in the urea cycle and is located primarily in liver cells. Arginase II (ArgII) functions in the

regulation of arginine/ornithine concentrations in cells and is found in mitochondria, kidney, prostate, macrophages, mammary glands and brain. Arginase inhibitors are known in the art and include any agent (small molecule or drug, antibody, soluble ligand, peptide, etc.) or analog or derivative thereof that can inhibit biological activity of arginase such that less arginine is catalyzed into ornithine and urea. Arginase inhibitors include, but are not limited to: nor-NOHA (N(omega)-Hydroxy-nor-L-arginine); NOHA (N^G-Hydroxy-L-arginine); BEC ((S)-(2-Boronoethyl)-L-cysteine); DMFO (DL-α-Difluoromethylornithine); ABH (2(S)-amino-6-boronohexanoic acid); H-Orn-OH·HCl; H-Ile-OH; L-valine; L-norvaline. Dosage of the arginine inhibitors is according to the manufacturer's directions.

Yeast-Based Immunotherapeutic Compositions Useful in the Invention

[0051] The present invention includes the use of at least one yeast-based immunotherapy composition. Additional immunotherapeutic compositions may also be used during the course of treatment of an individual according to the invention. Such additional immunotherapeutic compositions include, viral-based immunotherapy compositions, antibody-based immunotherapy compositions, DNA immunotherapy compositions, subunit vaccines, and any components or adjuvants useful for stimulating or modulating an immune response, such as TLR agonists, cytokines, immune potentiators, and other agents.

[0052] According to the invention, the phrase "yeast-based immunotherapeutic composition" (which phrase may be used interchangeably with "yeast-based immunotherapy product", "yeast-based composition", "yeast-based immunotherapeutic" or "yeast-based vaccine") 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 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, an immunotherapy 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. Optionally, a yeast-based immunotherapy composition is capable of eliciting a humoral immune response. A yeast-based immunotherapy composition useful in the present invention can, for example, elicit an immune response in an individual such that the

individual is treated for the disease or condition, or from symptoms resulting from the disease or condition.

[0053] Yeast-based immunotherapy compositions of the invention may be either "prophylactic" or "therapeutic". When provided prophylactically, the immunotherapy compositions of the present invention are provided in advance of any symptom of a disease or condition. The prophylactic administration of the immunotherapy compositions serves to prevent or ameliorate or delay time to onset of any subsequent disease. When provided therapeutically, the immunotherapy compositions are provided at or after the onset of a symptom of disease.

[0054] Typically, a yeast-based immunotherapy composition includes a yeast vehicle and at least one antigen or immunogenic domain thereof expressed by, attached to, or mixed with the yeast vehicle. In some embodiments, the antigen or immunogenic domain thereof is provided as a fusion protein. In one aspect of the invention, fusion protein can include two or more antigens. In one aspect, the fusion protein can include two or more immunogenic domains of one or more antigens, or two or more epitopes of one or more antigens. A TARMOGEN® is one non-limiting example of a yeast-based immunotherapy composition that is useful in the present invention. A TARMOGEN® (TARgeted MOlecular immunoGEN, 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. Tarmogens have been generally described in the art. See, e.g., U.S. Patent No. 5,830,463.

[0055] Yeast-based immunotherapy compositions, and methods of making and generally using the same, are described in detail, for example, in U.S. Patent No. 5,830,463, U.S. Patent No. 7,083,787, U.S. Patent No. 7,465,454, U.S. Patent Publication 2007-0224208, U.S. Patent Publication No. US 2008-0003239, and in 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. These yeast-based immunotherapeutic products have been shown to elicit immune responses, including cellular and humoral immune responses. Yeast-based immunotherapeutic products are capable of killing target cells expressing a variety of antigens in vivo, in a variety of animal species, and to do so via antigen-specific, CD4+ and CD8+ mediated immune responses. Additional studies have shown that yeast are avidly phagocytosed by and directly activate dendritic cells which then present yeast-

associated proteins to CD4+ and CD8+ T cells in a highly efficient manner. See, e.g., Stubbs et al. Nature Med. 5:625-629 (2001) and U.S. Patent No. 7,083,787.

[0056] In any of the yeast-based immunotherapy compositions used in the present invention, the following aspects related to the yeast vehicle are included in the invention. According to the present invention, a yeast vehicle 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 therapeutic composition of the invention, or in one aspect, the yeast vehicle can be used alone or as an adjuvant. The yeast vehicle can therefore include, but is not limited to, a live intact 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 thereof 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.

[0057] 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.

[0058] 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.

[0059] 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.

[0060] 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.

[0061] An example of a yeast cell wall preparation is 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.

[0062] 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, nonpathogenic yeast strains are used.

[0063] 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 due to it being 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. 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.

[0064] In one embodiment, a yeast vehicle of the present invention is capable of fusing with the cell type to which the yeast vehicle and antigen/agent is being delivered, such as a dendritic cell or macrophage, thereby effecting particularly efficient delivery of the yeast vehicle, and in many embodiments, the antigen(s) or other agent, to the cell type. As used herein, fusion of a yeast vehicle with a targeted cell type refers to the ability of the yeast cell membrane, or particle thereof, to fuse with the membrane of the targeted cell type (e.g., dendritic cell or macrophage), leading to syncytia formation. As used herein, a syncytium is a multinucleate mass of protoplasm produced by the merging of cells. A number of viral surface proteins (including those of immunodeficiency viruses such as HIV, influenza virus, poliovirus and adenovirus) and other fusogens (such as those involved in fusions between eggs and sperm) have been shown to be able to effect fusion between two membranes (i.e., between viral and mammalian cell membranes or between mammalian cell membranes). For example, a yeast vehicle that produces an HIV gp120/gp41 heterologous antigen on its surface is capable of fusing with a CD4+ T-lymphocyte. It is noted, however, that incorporation of a targeting moiety into the yeast vehicle, while it may be desirable under some circumstances, is not necessary. In the case of yeast vehicles that express antigens extracellularly, this can be a further advantage of the yeast vehicles of the present invention. In general, yeast vehicles useful in the present

invention are readily taken up by dendritic cells (as well as other cells, such as macrophages).

[0065] In most embodiments of the invention, the yeast-based immunotherapy composition includes at least one antigen, immunogenic domain thereof, or epitope thereof. The antigens contemplated for use in this invention include any antigen against which it is desired to elicit an immune response. In one embodiment the antigen is a cancer antigen.

Antigens Useful in the Yeast-Based Immunotherapy Compositions of the Invention

[0066] 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).

[0067] 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-based immunotherapeutic composition 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.

[0068] 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).

[0069] 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.

[0070] 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.

[0071] Antigens useful in any of the immunotherapy compositions described herein can include any antigen(s) or immunogenic domain(s) thereof against which it is desirable to elicit an immune response, and in particular, include any antigen(s) or immunogenic domain(s) thereof for which a therapeutic immune response against such antigen would be beneficial to an individual. The antigen can include, but is not limited to: a cancer antigen, a viral antigen, an overexpressed mammalian cell surface molecule, a bacterial antigen, a fungal antigen, a protozoan antigen, a helminth antigen, an ectoparasite antigen, a

mammalian cell molecule harboring one or more mutated amino acids, a protein normally expressed pre- or neo-natally by mammalian cells, a protein whose expression is induced by insertion of an epidemiologic agent (*e.g.* virus), a protein whose expression is induced by gene translocation, and a protein whose expression is induced by mutation of regulatory sequences.

[0072] In one aspect of the invention, antigens useful in one or more immunotherapy compositions of the invention include any cancer or tumor-associated antigen. In one aspect, the antigen includes an antigen associated with a preneoplastic or hyperplastic state. The antigen may also be associated with, or causative of cancer. Such an antigen may be a tumor-specific antigen, a tumor-associated antigen (TAA) or tissue-specific antigen, an epitope thereof, or an epitope agonist thereof. Cancer antigens include, but are not limited to, antigens from any tumor or cancer, including, but not limited to, melanomas, squamous cell carcinoma, breast cancers, head and neck carcinomas, thyroid carcinomas, soft tissue sarcomas, bone sarcomas, testicular cancers, prostatic cancers, ovarian cancers, bladder cancers, skin cancers, brain cancers, angiosarcomas, hemangiosarcomas, mast cell tumors, leukemias, lymphomas, primary hepatic cancers, lung cancers, pancreatic cancers, gastrointestinal cancers (including colorectal cancers), renal cell carcinomas and other kidney cancers, hematopoietic neoplasias and metastatic cancers thereof.

[0073] Suitable cancer antigens include but are not limited to carcinoembryonic antigen (CEA) and epitopes thereof such as CAP-1, CAP-1-6D (GenBank Accession No. M29540 or Zaremba et al., 1997, *Cancer Research* 57:4570-4577), MART-1 (Kawakami et al, *J. Exp. Med.* 180:347-352, 1994), MAGE-1 (U.S. Pat. No. 5,750,395), MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, MAGE-3, LAGE-1, BAGE-1, CTAG1, GAGE (U.S. Pat. No. 5,648,226), GP-100 (Kawakami et al., *Proc. Nat'l Acad. Sci. USA* 91:6458-6462, 1992), MUC-1 (*e.g.,* Jerome et al., *J. Immunol.*, 151:1654-1662 (1993)), MUC-2, mutated Ras oncoprotein (see, *e.g.,* U.S. Patent No. 7,465,454 and 7,563,447), normal and mutated p53 oncoproteins (Hollstein et al *Nucleic Acids Res.* 22:3551-3555, 1994), PSMA (prostate specific membrane antigen; Israeli et al., *Cancer Res.* 53:227-230, 1993), tyrosinase (Kwon et al *PNAS* 84:7473-7477, 1987), TRP-1 (gp75) (Cohen et al *Nucleic Acid Res.* 18:2807-2808, 1990; U.S. Pat. No. 5,840,839), NY-ESO-1 (Chen et al *PNAS* 94: 1914-1918, 1997), NY-BR-1, SCP-1, SSX-2, SSX-4, TRP-2 (Jackson et al., *EMBOJ*, 11:527-535, 1992), TAG72, KSA, CA-125, PSA (prostate specific antigen; Xue et al., *The Prostate,* 30:73-78 (1997)), HER-2/neu/c-erb/B2, (U.S. Pat. No. 5,550,214), EGFR

(epidermal growth factor receptor; Harris et al., *Breast Cancer Res. Treat*, 29:1-2 (1994)), hTERT, p73, B-RAF (B-Raf proto-oncogene serine/threonine-protein kinase; Sithanandam et al., (1990), *Oncogene* 5(12):1775–80), adenomatous polyposis coli (APC), Myc, von Hippel-Lindau protein (VHL), Rb-1, Rb-2, androgen receptor (AR), Smad4, MDR1 (also known as P-glycoprotein), Flt-3, carbonic anhydrase IX (CA9), BRCA-1 (breast cancer 1; U.S. Patent No. 5,747,282), BRCA-2 (breast cancer 2; U.S. Patent No. 5,747,282)), guanylyl cyclase C (GCC), Bcr-Abl, pax3-fkhr, ews-fli-1, Brachyury (GenBank Accession Nos. NP_003172.1 or NM_003181.2; Edwards et al., 1996, *Genome Res.* 6:226-233), HERV-H (human endogenous retrovirus H), HERV-K (human endogenous retrovirus K) including but not limited to HERV-K-MEL, TWIST (GenBank Accession Nos. NM_000474 and NP_000465), Mesothelin (Kojima et al., 1995, *J. Biol. Chem.* 270(37):21984–90; Chang and Pastan, 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93(1):136–40), NGEP (New Gene Expressed in Prostate; Bera et al., 2004, *Proc. Natl. Acad. Sci. U.S.A.* 101(9):3059-3064; Cereda et al., 2010, *Cancer Immunol. Immunother.* 59(1):63-71; GenBank Accession Nos. AAT40139 or AAT40140), modifications of such antigens and tissue specific antigens, splice variants of such antigens, and/or epitope agonists of such antigens. Other cancer antigens are known in the art. Other cancer antigens may also be identified, isolated and cloned by methods known in the art such as those disclosed in U.S. Pat. No. 4,514,506. Cancer antigens may also include one or more growth factors and splice variants of each. In one embodiment, the cancer antigen used in a yeast-based immunotherapy product is a human cancer antigen (an antigen found in human cancer cells or expressed in humans).

[0074] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with a brain cancer, including but not limited to, glioblastoma multiforme. Such antigens include, but are not limited to: epidermal growth factor receptor (EGFR) including EGFRvIII, mutated Ras (mutations at positions 12, 13, 59 and/or 61), mitogen-activated protein kinase (MAPK), interleukin-13 receptor-α2 (IL-13Rα2), gp100, TRP-2, MAGE-A3, MAGE-1, Her-2/neu, EphA2, survivin, Wilm's Tumor 1 (WT1), Sry-Related High-Mobility Group Box-2 (SOX2), SOX11, AIM2, and Squamous Cell Carcinoma Antigen Recognized by T Cells 1 (SART1). See, *e.g.,* Okada et al., 2009, *Crit. Rev. Immunol.* 29(1):1-42 or Vauleon et al., 2010, *Clin. Dev. Immunol.* Epub pii.689171 for a review of these antigens.

[0075] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with a lung cancer, including but not limited to, non-small cell lung cancer. Such antigens include, but are not limited to: mutated Ras (mutations at positions 12, 13, 59 and/or 61), carcinoembryonic antigen (CEA), mucin-1 (MUC1), Brachyury, EGFR, Her2/neu, NY-ESO-1, MAGE-A3, and EGFR.

[0076] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with a renal cell cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, multidrug resistance-associated protein 3, polycomb group protein enhancer of zeste homologue 2, Her2/neu, von Hippel-Lindau (VHL) protein, kidney cancer antigen 1 (KCAG1), TRP-2, FGF-5, and renal cell carcinoma-associated antigen G250.

[0077] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with breast cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, TWIST, hTERT, Her2/neu, NY-BR-1, EphA2, CTAG1, carbonic anhydrase IX (CA9), MAGE-A3, MAGE-1, BAGE-1, HERV-K and HERV-H.

[0078] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with pancreas cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, LAGE-1, NY-ESO-1, SCP-1, SSX-2, SSX-4, HERV-K-MEL and HERV-H.

[0079] In one aspect of the invention, the antigen to be expressed by the yeast is an antigen associated with colorectal cancer. Such antigens include, but are not limited to: CEA, MUC1, Brachyury, mutated Ras, EGFR, CTAG1, MAGE-A3, MAGE-1, BAGE1, GCC, Her2/neu, carbonic anhydrase IX (CA9), HERV-K and HERV-H.

[0080] In another aspect of the invention, antigens useful in one or more immunotherapy compositions of the invention include any antigens associated with a pathogen or a disease or condition caused by or associated with a pathogen. Such antigens include, but are not limited to, viral antigens, fungal antigens, bacterial antigens, helminth antigens, parasitic antigens, ectoparasite antigens, protozoan antigens, or antigens from any other infectious agent.

[0081] In one aspect, the antigen is from virus, including, but not limited to, adenoviruses, arena viruses, bunyaviruses, coronaviruses, coxsackie viruses, cytomegaloviruses, Epstein-Barr viruses, flaviviruses, hepadnaviruses, hepatitis viruses,

herpes viruses, influenza viruses, lentiviruses, measles viruses, mumps viruses, myxoviruses, orthomyxoviruses, papilloma viruses, papovaviruses, parainfluenza viruses, paramyxoviruses, parvoviruses, picornaviruses, poxviruses, rabies viruses, respiratory syncytial viruses, reoviruses, rhabdoviruses, rubella viruses, togaviruses, and varicella viruses. Other viruses include T-lymphotrophic viruses, such as human T-cell lymphotrophic viruses (HTLVs, such as HTLV-I and HTLV-II), bovine leukemia viruses (BLVS) and feline leukemia viruses (FLVs). The lentiviruses include, but are not limited to, human (HIV, including HIV-1 or HIV-2), simian (SIV), feline (FIV) and canine (CIV) immunodeficiency viruses. In one embodiment, viral antigens include those from non-oncogenic viruses. In one embodiment, the viruses are hepatitis C virus (HCV) or hepatitis B virus (HBV).

[0082] In another aspect, the antigen is from an infectious agent from a genus selected from: *Aspergillus, Bordatella, Brugia, Candida, Chlamydia, Coccidia, Cryptococcus, Dirofilaria,* Escherichia, *Francisella, Gonococcus, Histoplasma, Leishmania, Mycobacterium, Mycoplasma, Paramecium, Pertussis, Plasmodium, Pneumococcus, Pneumocystis, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Toxoplasma, Vibriocholerae*, and *Yersinia*. In one aspect, the infectious agent is selected from *Plasmodium falciparum* or *Plasmodium vivax*.

[0083] In one aspect, the antigen is from a bacterium from a family selected from: Enterobacteriaceae, Micrococcaceae, Vibrionaceae, Pasteurellaceae, Mycoplasmataceae, and Rickettsiaceae. In one aspect, the bacterium is of a genus selected from: *Pseudomonas, Bordetella, Mycobacterium, Vibrio, Bacillus, Salmonella, Francisella, Staphylococcus, Streptococcus, Escherichia, Enterococcus, Pasteurella,* and *Yersinia*. In one aspect, the bacterium is from a species selected from: *Pseudomonas aeruginosa, Pseudomonas mallei, Pseudomonas pseudomallei, Bordetella pertussis, Mycobacterium tuberculosis, Mycobacterium leprae, Francisella tularensis, Vibrio cholerae, Bacillus anthracis, Salmonella enteric, Yersinia pestis, Escherichia coli* and *Bordetella bronchiseptica*.

[0084] In one aspect, the antigen is from a fungus, such a fungus including, but not limited to, a fungus from Saccharomyces *spp., Aspergillus spp., Cryptococcus spp., Coccidioides spp., Neurospora spp., Histoplasma spp.,* or *Blastomyces* spp.. In one aspect, the fungus is from a species selected from: *Aspergillus fumigatus, A. flavus, A. niger, A. terreus, A. nidulans, Coccidioides immitis, Coccidioides posadasii* or *Cryptococcus neoformans*. The most common species of *Aspergillus* causing invasive disease include *A.*

fumigatus, A. flavus, A. niger, A. terreus and *A. nidulans*, and may be found, for example, in patients who have immunosuppression or T-cell or phagocytic impairment. *A. fumigatus* has been implicated in asthma, aspergillomas and invasive aspergillosis. Coccidioidomycosis, also known as San Joaquin Valley Fever, is a fungal disease caused by *Coccidioides immitis*, and can lead to acute respiratory infections and chronic pulmonary conditions or dissemination to the meninges, bones, and joints. Cryptococcosis-associated conditions are also targeted by methods of the invention, for example, in a non-immunosuppressed or immunosuppressed subject, such as a subject who is infected with HIV.

[0085] In some embodiments, the antigen is a fusion protein. In one aspect of the invention, a fusion protein can include two or more antigens. In one aspect, the fusion protein can include two or more immunogenic domains and/or two or more epitopes of one or more antigens. Any combination of antigens, immunogenic domains thereof, and/or epitopes thereof are contemplated for use in the compositions of the invention. An immunotherapeutic composition containing such antigens, immunogenic domains thereof, and/or epitopes thereof may provide antigen-specific immunization in a broad range of patients. For example, a fusion protein useful in the present invention may have multiple domains (two or more domains), wherein each domain consists of a peptide or polypeptide from a particular protein, the peptide or polypeptide consisting of at least 4 amino acid residues flanking either side of and including a mutated amino acid that is found in the protein, wherein the mutation is associated with a particular disease or condition.

[0086] Optionally, proteins, including fusion proteins, which are used as a component of the yeast-based 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.

[0087] 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.

[0088] 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:1). 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.

[0089] In one embodiment, the 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:2 or SEQ ID NO:3. Other sequences for yeast alpha factor prepro sequence are known in the art and are encompassed for use in the present invention.

[0090] 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.

[0091] 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).

[0092] 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.

[0093] 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.

[0094] 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.

[0095] 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.

[0096] 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.

[0097] 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.

[0098] 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.

[0099] 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.

[00100] 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.

[00101] Transcription control sequences to express genes in methyltrophic yeast include the transcription control regions of the genes encoding alcohol oxidase and formate dehydrogenase.

[00102] 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.

[00103] 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 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 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.

[00104] One non-limiting example of a medium suitable for the culture of a yeast-based immunotherapy composition of the invention is U2 medium. U2 medium comprises the following components: 20g/L of glucose, 6.7 g/L of Yeast nitrogen base containing ammonium sulfate, and 0.04 mg/mL each of histidine, leucine, tryptophan, and adenine. Another non-limiting example of a medium suitable for the culture of yeast-based immunotherapy composition of the invention is UL2 medium. UL2 medium comprises the following components: 20g/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.

[00105] 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. 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)..

[00106] 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. In one aspect, glycosylation of the antigen is desirable and such strains and methods are not utilized.

[00107] 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.

[00108] 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.

[00109] 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.

[00110] 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).

[00111] 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.

[00112] 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.

[00113] 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.

[00114] 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).

[00115] 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.

[00116] 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.

[00117] 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 and/or agents of the invention to prevent or treat a disease, including cancer, that is associated with or characterized by MDSC-mediated or arginase-mediated immunosuppression. In one aspect, the kit includes a yeast-based immunotherapeutic composition and an arginine supplement.

Methods for Administration or Use of Compositions and Therapies of the Invention

[00118] As discussed above, the invention generally relates to a method to treat and/or prevent a disease characterized by MDSC-mediated or arginase-mediated immunosuppression, which includes the steps of administering to an individual with the disease a yeast-based immunotherapy composition and arginine therapy. The yeast-based immunotherapy composition and the arginine therapy may be administered in combination, concurrently, sequentially, or in any combination of these types of administration.

[00119] The yeast-based immunotherapy composition and arginine therapy can be administered in combination (*e.g.,* together in the same composition), concurrently (*e.g.,* over the same period of time, although not necessarily in the same composition), sequentially, or in alternating manner, or any combination of these protocols. As used herein with respect to administration of a yeast-based immunotherapy composition and arginine therapy, the term "concurrently" means to administer each of the compositions/agents essentially at the same time or within the same dosing period, or within a time period during which the effects of priming of the immune system by the immunotherapy composition occurs (*e.g.*, within 1-2 days and preferably less). For clarity, concurrent administration does not require administration of all of the compositions/agents at precisely the same moment, but rather, the administration of all compositions/agents should occur within one scheduled dosing of the patient in order to prime the immune system with each of the compositions concurrently (*e.g.,* one composition/agent may be administered first, followed immediately or closely by the administration of the second composition/agent, and so on).

[00120] In one aspect of the invention, arginine therapy is administered sequentially with the yeast-based immunotherapy composition. In another embodiment, arginine therapy is administered before the yeast-based immunotherapy composition is administered. In another embodiment, arginine therapy is administered after the yeast-based immunotherapy composition is administered. In one embodiment, arginine therapy is administered in alternating doses with the yeast-based immunotherapy composition, or

in a protocol in which the yeast-based immunotherapy composition is administered at prescribed intervals in between or with one or more consecutive doses of arginine therapy, or *vice versa*. In one embodiment, the yeast-based immunotherapy composition is administered in one or more doses over a period of time prior to commencing the administration of arginine therapy. In other words, the yeast-based immunotherapeutic composition is administered as a monotherapy for a period of time, and then arginine therapy is added, either concurrently with new doses of immunotherapy, or in an alternating fashion with immunotherapy. Alternatively, arginine therapy may be administered for a period of time (as a monotherapy) prior to beginning administration of the yeast-based immunotherapy composition, and then yeast-based immunotherapy is added, either concurrently with new doses of arginine therapy, or in an alternative fashion with arginine therapy. In addition, these concepts may be combined with other therapeutic approaches for the treatment of a disease (*e.g.,* surgical resection of a tumor, chemotherapy, radiation, T cell adoptive transfer, small molecule treatment, other immunotherapy), provided sequentially, concurrently, or in alternating fashion. In one embodiment, the arginine therapy will be administered more frequently than the yeast-based immunotherapy composition. These aspects of the invention are not mutually exclusive. For example, a clinician may prescribe arginine therapy prior to use of yeast-based immunotherapy, with continuation of arginine therapy during yeast-based immunotherapy, and optionally, with continuation of arginine therapy for a period of time after yeast-based immunotherapy. Various protocols for the treatment of disease, including cancer, using yeast-based immunotherapy and arginine therapy are contemplated by the invention, and these examples should be considered to be non-limiting examples of various possible protocols.

[00121] Various methods of the invention treat a disease or condition by administering compositions of the invention. As used herein, the phrase "treat a disease", or any permutation thereof (*e.g.,* "treated for a disease", etc.) can generally refer to preventing a disease, preventing at least one symptom of the disease, delaying onset of a disease, reducing one or more symptoms of the disease, reducing the occurrence of the disease, and/or reducing the severity of the disease. 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 the target antigen 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). With respect to infectious disease and other diseases, the methods of the invention can result in one or more of: prevention of the disease or condition, prevention of infection, delay the onset of disease or symptoms caused by the infection, increased survival, reduction of pathogen burden (*e.g.,* reduction of viral titer), reduction in at least one symptom resulting from the infection in the individual, reduction of organ or physiological system damage resulting from the infection or disease, improvement in organ or system function, and/or improved general health of the individual.

[00122] The present invention includes the delivery (administration, immunization) of a composition of the invention to a subject. 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 be returned to a patient, or administered to a patient, by any suitable mode of administration.

[00123] Administration of a composition can be systemic, local, mucosal and/or proximal to the location of the target site (e.g., near a site of infection). Suitable routes of administration will be apparent to those of skill in the art, depending on the type of condition to be prevented or treated, the antigen used, 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, subcutaneous 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, which is incorporated herein by reference in its entirety). Other routes of administration that modulate mucosal immunity are useful in the treatment of viral infections. Such routes include bronchial, intradermal, intramuscular, intranasal, other inhalatory, rectal, subcutaneous, topical, transdermal, vaginal and urethral routes. In one aspect, an immunotherapeutic composition of the invention is administered subcutaneously.

[00124] A dosage of arginine (*e.g.,* arginine supplementation), including L-arginine or any derivative or analog thereof, and including any form of arginine described herein or otherwise known in the art, for use in the present invention is typically a dose of from about 5 mg per day to about 5 grams per day, including any intermediate whole integer dosage in 1 mg increments (*i.e.,* 5 mg/day, 6 mg/day, 7 mg/day, etc.). In one aspect of the invention, the dose of arginine is 15 mg/day, 16, mg/day, 17 mg/day, 18 mg/day, 19

mg/day, 20 mg/day, 21 mg/day, 22 mg/day, 23 mg/day, 24 mg/day, 25 mg/day, 26 mg/day, 27 mg/day, 28 mg/day, 29 mg/day, or 30 mg/day. In one aspect, the dose of arginine is between 20 and 30 mg per day, or between 20 and 25 mg per day. In one aspect, the dose of arginine is 24 mg per day. A daily dose of arginine can be divided among two, three, four, or more sub-doses administered at intervals during the day, or a daily dose of arginine can be administered in a single dose daily. In one embodiment, the daily dose is divided among four equivalent doses in a day (*e.g.,* 6 mg four times daily for a total of 24 mg/day). Doses of arginine can be administered on a different schedule than daily, including every other day, every three days, every four days, every five days, every six days, or weekly, bi-weekly, or monthly.

[00125] A suitable dose of an arginase inhibitor, including any of the arginase inhibitors as described herein or as known in the art, will vary from inhibitor to inhibitor. Guidelines for administration of arginase inhibitors are provided by the manufacturer of the inhibitor, but should be an effective dose to decrease the biological activity of arginase in an individual, such that the activity of a yeast-based immunotherapy composition is improved (as measured by improvements in immune responses, and particularly T cell responses, or as measured by improvements in survival, reductions in tumor or infectious disease burden, or as measured by improvements of another measure of efficacy of the yeast-based immunotherapy composition in the given disease), as compared to in the absence of the arginase inhibitor.

[00126] In general, a suitable single dose of a yeast-based immunotherapeutic composition is a dose that is capable of effectively providing a yeast vehicle and the target 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 target 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-based immunotherapy composition 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-based 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.

[00127] "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 from about 1, 2, 3, 4, 5, 6, 7, or 8 weeks apart, to monthly, to bimonthly, to quarterly, to annually, to several years after the original administration. In one embodiment, an administration schedule is one in which from about 1 x 10^5 to about 5 x 10^7 yeast cell equivalents of a composition per kg body weight of the organism 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.

[00128] In the method of the present invention, compositions and therapeutic compositions can be administered to 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 protect include humans, dogs, cats, mice, rats, goats, sheep, cattle, horses and pigs.

[00129] 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

[00130] 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 and W. Orenstein, eds., 3rd edition (1999).

[00131] An "immunotherapeutic composition" is a composition that elicits an immune response sufficient to achieve at least one therapeutic benefit in a subject.

[00132] In general, the term "biologically active" indicates that a compound has at least one detectable activity that has an effect on the metabolic or other processes of a cell or organism, as measured or observed in vivo (i.e., in a natural physiological environment) or in vitro (i.e., under laboratory conditions).

[00133] Reference to an isolated protein or polypeptide in the present invention includes full-length proteins, fusion proteins, or any fragment, domain, conformational epitope, or homologue of such proteins. More specifically, 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.

[00134] As used herein, the term "homologue" is used to refer to a protein or peptide which differs from a naturally occurring protein or peptide (i.e., the "prototype" or "wild-type" protein) by minor modifications to the naturally occurring 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 can have either enhanced, decreased, or substantially similar properties as compared to the naturally occurring protein or peptide. A homologue can include an agonist of a protein or an antagonist of a protein. Homologues can be produced using techniques known in the art for the production of proteins including, but not limited to, direct modifications to the isolated, naturally occurring 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.

[00135] A homologue 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 90%, or at least about 95% 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. In one embodiment, the homologue 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 naturally occurring amino acid sequence of the reference protein.

[00136] As used herein, unless otherwise specified, reference to a percent (%) identity refers to an evaluation of homology which is performed using: (1) a BLAST 2.0 Basic BLAST 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 (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 2 alignment (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 BLAST 2.0 Basic BLAST and BLAST 2, two specific sequences might be recognized as having significant homology using the BLAST 2 program, whereas a search performed in BLAST 2.0 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.

[00137] Two specific sequences can be aligned to one another using BLAST 2 sequence 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. BLAST 2 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 2 sequence alignment 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).

[00138] 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 in which the nucleic acid molecule is found in nature. An isolated nucleic acid molecule can include a 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 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.

[00139] 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.

[00140] 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 virus or a plasmid. 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.

[00141] 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.

[00142] 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.

[00143] The following experimental results are provided for purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

[00144] The following example demonstrates that patients with glioblastoma multiforme (GBM) harbor significantly expanded populations of circulating MDSC associated with increased levels of serum arginase I (ArgI), and that targeting ArgI-mediated immunosuppression using arginine therapy (by arginine supplementation or by inhibition of arginase) results in complete restoration of GBM T cell functional responses.

[00145] To define the source of immunosuppression in patients with GBM, the inventors recently identified an expanded population of cells within the peripheral blood of GBM patients that express a pattern of markers previously defined as characteristic of MDSC (data not shown). These cells, phenotypically defined as CD11b+/CD33lo/HLA-DRneg/CD14neg, are not found at increased frequency in patients with non-malignant brain tumors (data not shown). Additional studies showed that the MDSC within GBM patients express the canonical neutrophil (granulocytic) markers, CD15 and CD66.

[00146] *Materials and Methods.* For the experiments described in this example, peripheral blood was collected from patients undergoing neurosurgical resection of intracranial tumors (GBM, anaplastic glioma, meningioma, and pituitary tumor) at the University of Colorado Hospital. Patient age and gender did not vary significantly between groups. Normal donor blood was collected from anonymous donors from the blood bank at the University of Colorado. Within one hour from harvest, plasma was removed from peripheral blood samples and stored at -70°C. Peripheral blood mononuclear cells (PBMC) were purified from patients and normal donors by centrifugation over a Ficoll Histopaque (Sigma) density gradient according to the manufacturer's protocol. PBMC were used immediately, without freezing, for T cell functional assays.

[00147] For T cell functional assays, phytohemagglutinin (PHA) (Sigma) stimulations were performed using bulk PBMC or isolated T cells from normal donors or patients

cultured in RPMI 1640 media with 10% FBS and 1% penicillin-streptomycin. T cells were isolated using CD3 positive selection magnetic beads per manufacturer's protocol (Miltenyi Biotec). Cells were plated at a concentration of $1x10^5$ cells per well in a 48 well plate with 500 μL of media per well. PHA was added at a concentration of 5μg/mL to each well and plates were incubated for 48 and 72 hours post-PHA stimulation. Media interferon- γ (IFN-γ) levels were assayed by ELISA (Thermo Scientific) according to the manufacturer's protocol. Mixed lymphoid reactions (MLR) were carried out using bulk PBMC collected from patients and normal donors. "Modified" MLR utilized purified CD11b+ myeloid cells and CD3+ T cells from patients and normal donors, again isolated using positive bead selection (Miltenyi Biotec). Cells from two different normal donors or a normal donor and a tumor patient were mixed at a concentration of $1.0x10^5$ cells/well per cell type in 200 μL RPMI 1640 with 10% FBS and 1% pen-strep in 96 well plates. MLR were incubated for 48 and 72 hours and media IFN-γ levels were assayed by ELISA as described above.

[00148] For measurement of arginase I (ArgI), plasma samples, collected from patients or normal donors, as well as media from T cell functional assays described above were subject to Arginase I ELISA (Hycult Biotechnology) according to the manufacturer's protocol. Plasma samples were diluted 1:1 with kit dilution buffer prior to incubation.

[00149] For evaluation of reversal of arginase activity *in vitro,* T cell functional assays (PHA stimulation, MLR, and "modified" MLR) using either bulk PMBC or isolated T cells and myeloid cells were performed as above. Groups of samples were treated with 7.81 μg/mL L-arginine (Sigma) or 40 μM nor-NOHA (Cayman Chemicals) added to media at the time of plating and PHA addition or cell mixing. Dose-response profiles for each compound were developed prior to testing on patient samples, in order to identify the highest possible dose that did not affect baseline T cell functional response (*i.e.* toxicity or augmented functional response) in normal donor samples (data not shown). Cells were incubated for 48 and 72 hours, and media IFN-γ levels were tested by ELISA as described above.

[00150] Data are represented as mean ± SEM. Multigroup analysis was performed using ANOVA. Differences between two variables were determined using Student's *t* test. P values less than 0.05 were considered significant.

[00151] *Results.* Studies by one of the present inventors have now confirmed that T cells from GBM patients demonstrate minimal proliferation and IFN-γ production upon

activation directly *ex vivo*, and importantly, have documented increased numbers of degranulated neutrophils within the peripheral circulation of patients with GBM, which is associated with increased levels of ArgI in plasma (data not shown). To explore potential *in vivo* activity of these MDSC, serum samples from patients with GBM were evaluated by ELISA for increased levels of ArgI, and found to bear nearly 3-fold higher levels of ArgI than were seen in sera from normal donors or patients with intracranial meningioma or metastasis (Fig. 1). These data show that patients with GBM harbor significantly expanded populations of circulating MDSC associated with increased levels of serum ArgI.

[00152] Since ArgI is known to regulate CD3ζ expression by T cells, the levels of this marker on unmanipulated, direct *ex vivo* T cells from GBM patients was evaluated. Flow cytometric analysis demonstrated that baseline CD3ζ levels were indeed significantly lower upon circulating T cells from GBM patients than T cells from patients with pituitary tumors or meningioma (data not shown).

[00153] In order to demonstrate that the immunosuppressive effect of ArgI release and further confirm that the proposed mechanism of MDSC-mediated effects could be recapitulated *in vitro*, media harvested from PHA-stimulated PBMC from GBM patients (GBM) was evaluated for ArgI. As shown in Fig. 2A, PHA-stimulated PBMC from GBM patients contained significantly higher levels of ArgI than cultures of PBMC from normal donors (ND) or patients with meningioma (MEN) (Fig. 2A). The same phenomenon was observed when sorted MDSC from patients with GBM were used in a "modified MLR" (Fig. 2B). Indeed, the results showed that media harvested from GBM-associated MLR and mitogen-stimulated GBM T-cell cultures contained ArgI levels nearly 5-fold higher than within media from control cultures (Fig. 2A and Fig. 2B). These data complete a mechanistic linkage between circulating MDSC, elevated levels of serum ArgI, and T cell abnormalities that are characteristically induced by MDSC within GBM patients. Therefore, the inventors conclude that GBM has the capacity to induce the expansion of ArgI-expressing MDSC *in vivo* and that the MDSC-ArgI pathway contributes to the suppression of cellular immunity seen in these patients.

[00154] Confirmation of MDSC-mediated immunosuppression in GBM patients was provided by *in vitro* experiments designed to rescue ArgI-derived dysfunction of T cells. Using the aforementioned functional assays with either mitogen stimulation or MLR, culture media was augmented with the specific ArgI antagonist, nor-NOHA, or supplemental L-arginine at physiological levels, as described above. The results

demonstrated that IFN-γ production was significantly limited in the presence of MDSC from GBM in both PHA-stimulated cultures (Fig. 2C) and MLR (Fig. 2D). However, this effect can be *completely reversed* in both settings by using the selective ArgI inhibitor nor-NOHA or through the addition of supplemental L-arginine. More specifically, both reagents provided complete restoration of IFN-γ production by mitogen-stimulated GBM T cells or ND T cells in the presence of MDSC from GBM (Figs. 2C and 2D). Together, these results provide confirmation that MDSC-mediated suppression of cellular immunity in GBM patients is reversible and can be improved by targeting the effects of MDSC-derived ArgI.

[00155] These data lead to several key conclusions regarding immunosuppression in patients with GBM: (1) Phenotypic and functional abnormalities within circulating T cells confirm that GBM-specific immunosuppressive effects extend beyond the local brain environment, mandating the presence of an intermediary immunosuppressive agent; (2) Patients with GBM harbor significantly increased numbers of circulating MDSC that are associated with increased levels of serum ArgI; (3) Sorted MDSC from patients with GBM can suppress normal donor T cell responses through an ArgI-specific mechanism, providing functional confirmation for suppressive activity of these cells *in vivo*; and (4) Targeting ArgI-mediated immunosuppression *in vitro* results in complete restoration of GBM T cell functional responses.

[00156] These conclusions contributed to the innovative approach of the present invention: targeting MDSC-mediated immunosuppression to restore cellular immune function and augment the efficacy of immunotherapeutic strategies, such as yeast-based immunotherapy, for patients with GBM and other cancers and diseases associated with MDSC-mediated immunosuppression.

Example 2

[00157] The following experiment demonstrates that arginine therapy augments endogenous anti-tumor immunity by targeting tumor-derived MDSCs, thereby enabling the additional enhancement of immunotherapy approaches using yeast-based immunotherapy.

[00158] This experiment expands upon prior data demonstrating the generation of MDSC in the rat glioma model. Prior studies have shown that rat GBM, after stereotactic implantation and growth of tumors within the brain of adult rats, results in the expansion of CD11bc+/Gr-1+ MDSC in the periphery and within tumors. These cells have been

directly associated with increased Arg1 expression and immunosuppression in tumor-bearing animals, and the suppressive effect can be transferred to lymphocytes from non-tumor bearing hosts (Graf, 2005; Jia, 2010). As described in Example 1, the inventors have now shown that the cellular immune function in GBM patients can be completely restored through the use of ArgI inhibitors or through simple arginine supplementation. Since L-arginine is an amino acid that is readily available commercially, and is inexpensive and well-tolerated when orally administered, the clinical benefits and potential for rapid translation to use in cancer patients are enormous. Therefore, the following experiment is to provide pre-clinical *in vivo* testing of oral arginine for improving MDSC-related immunosuppression in the setting of rat GBM, in advance of utilizing this approach in combination with yeast-based immunotherapy. Specifically, these data will: 1) provide a temporal and quantitative framework for the timing of MDSC generation following tumor implantation, 2) quantitate the extent of suppression exerted upon global cellular immune responses following tumor implantation, and 3) evaluate the efficacy of targeting MDSC-derived immunosuppression for improving endogenous cellular immunity in the 9L-EGFR model.

[00159] Female adult Fischer rats are intracerebrally inoculated with 1 x 10^5 9L-EGFR rat glioma cells by stereotactic injection into the deep right frontal white matter, as previously described (Lopez, 2006). Based on experience with wild-type as well as EGFR-9L cells in this model, animals predictably die from tumor-associated mass effect and neurological decline within 17-25 days post-injection. Use of the EGFR-9L line also allows for subsequent specific vaccination using an antigen that is one of the most commonly encountered variants in GBM (see Examples 3 and 4).

[00160] ***MDSC generation in the 9L-EGFR model:*** Groups of three animals are sacrificed post-injection at days 7 (the time at which gross tumor can typically be first appreciated), 11, 16, and 20 (or at the time of euthanasia due to symptomatic decline, whichever is first). A group of sham animals undergoes anesthesia and needle insertion but are sacrificed immediately after the procedure to serve as surgical/baseline controls. Blood is harvested by cardiac puncture and used for preparation of mononuclear cells (PBMCs) using Ficoll density centrifugation. Serum from blood samples is used for quantitative ArgI detection via ELISA as described in Example 1. Splenic and lymphoid tissues are harvested and mononuclear cells are extracted as per common protocols. Brains are harvested and tumor tissue is dissected and reduced to single-cell suspension as

previously described (Waziri, 2008). A portion of the resultant cell preps from the aforementioned tissues along with matched PBMCs are stained for flow cytometry to identify frequencies of CD4+ and CD8+ T cells as well as the frequency of CD11bc+/His48+ (BD Biosciences) MDSC in each compartment. T cell and MDSC ratios for each tissue are compiled and compared across the various time points tested. Subsets of cells are prepared for intracellular cytokine staining for IFN-γ, IL-13, and IL-17, as previously described, in order to detect direct *ex vivo* T cell functional biasing (Waziri, 2008). Quantitative data for T cell phenotypes and frequencies from within peripheral and tumor tissues are used as baseline data for subsequent analysis regarding improvement in tumor immune infiltrates in response to arginine supplementation and vaccination. The results of these initial analyses show a linear and reproducible increase in circulating MDSC with an associated decrease in T cell functional responses associated with tumor growth.

[00161] ***Functional assesment of MDSC-derived immunosuppression in the 9L-EGFR model:*** To provide quantitative data regarding MDSC suppressive activity in untreated tumor-bearing animals, PBMCs obtained in parallel to the experiments outlined above are used for T cell functional assays. Cells (1×10^6) are labeled with CFSE and then subjected to Concanavalin A (ConA)-induced stimulation for 48, 72, and 96 hours. Proliferative responses are evaluated via CSFE dilution and media is used for IFN-γ ELISA. Potential immunosuppressive effects on T cell proliferation are quantitiated by comparative analysis between sequential time points throughout the clinical course, comparing the ratio of proliferating T cells within ConA-stimulated cells harvested from animals. In parallel, T cell capacity for IFN-γ production will be quantitatively compared. ConA-stimulated cells will be generated as above and media will be harvested at 48 and 72 hours for use in IFN-γ ELISA analysis. T cell functional responses, as measured by ConA-induced proliferation and IFN-γ production, are expected to decrease in correlation with tumor growth and expansion of circulating MDSC. These experiments provide detailed baseline data regarding the temporal development and extent of immunosuppressive effects for MDSC generated by the 9L-EGFR tumor model.

[00162] ***Targeting MDSC-mediated immunosuppression using oral Arg or intraperitoneal nor-NOHA:*** Following intracranial tumor implantation, groups of animals are treated with oral arginine supplementation (Sigma) or intraperitoneal (IP) nor-NOHA (an ArgI antagonist; Sigma) to target MDSC-derived ArgI based

immunosuppression. Based upon prior studies demonstrating safety and efficacy of arginine supplementation in rats, several doses are utilized. Starting on the day of tumor inoculation, solutions of 0.5%, 2%, and 5% arginine in water are provided to study animals *ad libitum*. Additional groups of study animals receive daily IP injection of 10, 50, or 100ug of nor-NOHA in 100ul saline vehicle, to begin on day 1 following tumor implantation. For survival analyses and evaluation of potential therapeutic benefit of targeting ArgI activity in isolation (*i.e.,* without vaccination), initial treatment and control groups are observed through the entirety of their clinical course for the purposes of generating survival data. In parallel, groups of animals in each treatment arm are sacrificed at the timepoints outlined above. Phenotypic and functional evaluation of T cells is performed as described above to document treatment-associated improvements in MDSC-related immunosuppression. In order to detect increased endogenous anti-tumor immune responses, T cell infiltration of tumors is assessed by flow cytometry and compared to baseline data acquired from studies described above. Treatment with oral Arg or nor-NOHA i.p. is expected to result in augmented cellular immune function in tumor-bearing animals when compared to controls and further to increase the extent and clinical effect of endogenous anti-tumor immunity.

Example 3

[00163] The following example demonstrates the use of yeast-based immunotherapy comprising a human cancer antigen to treat a brain tumor *in vivo*.

[00164] ***Targeting Brain Tumors Using Yeast-Based Immunotherapy:*** Vaccines that simultaneously deliver target antigens to dendritic cells (DC) and promote DC maturation and activation offer the significant advantage of providing both MHC class I and class II T cell stimulation. Yeast-based immunotherapy achieves both goals for activating cell-mediated immune responses. Yeast-EGFR immunotherapy (whole, heat-killed recombinant yeast expressing EGFR) described herein expresses an inactive form of the human EGFR gene product as a soluble cytosolic polypeptide, and the whole heat-killed yeast are then administered as the vaccine.

[00165] Four different yeast-EGFR constructs were prepared for use in various experiments described below, as follows.

[00166] Yeast (*Saccharomyces cerevisiae*) were engineered to express human or rat epidermal growth factor receptor (EGFR) to produce yeast-EGFR immunotherapy compositions, denoted GI-3001 (human EGFR), GI-3005 (rat EGFR), GI-3006 (human

EGFR) and GI-3010 (human EGFR). The EGRF antigens expressed in each of GI-3001, GI-3005 and GI-3006 are under the control of the copper-inducible promoter, *CUP1*, and the EGFR antigen expressed by GI-3010 is under the control of the constitutive promoter, *TEF2*.

[00167] For the yeast-based immunotherapeutics referred to as GI-3006 and GI-3010, the yeast recombinantly express a human EGFR antigen as a single polypeptide, represented by SEQ ID NO:4. As compared to full-length, wild-type human EGFR, SEQ ID NO:5 lacks the N-terminal signal sequence and transmembrane domain of full-length human EGFR (*e.g.,* as compared to GENBANK® Accession No. NM005228), and is therefore expressed as a soluble, cytosolic protein in the yeast. SEQ ID NO:5 is encoded by a nucleotide sequence represented by SEQ ID NO:4, which has been codon-optimized for expression in yeast. The yeast-based immunotherapeutic known as GI-3001 also expresses a human EGFR protein which is nearly identical to that of SEQ ID NO:5, except for single amino acid substitutions at position 173 (G to S) and position 413 (V to A), relative to SEQ ID NO:5. The yeast-based immunotherapeutic known as GI-3005 expresses a rat EGFR protein having the amino acid sequence of SEQ ID NO:7. As compared to full-length, wild-type rat EGFR, SEQ ID NO:7 lacks the N-terminal signal sequence and the transmembrane domain, and is therefore also expressed as a soluble cytosolic protein by the yeast. SEQ ID NO:7 is encoded by a nucleotide sequence represented by SEQ ID NO:6, which has been codon-optimized for expression in yeast.

[00168] To prepare the yeast-EGFR immunotherapeutic compositions, DNA encoding the EGFR proteins was amplified using PCR, and then inserted at cloning sites behind the *CUP1* promoter or the *TEF2* promoter in yeast 2 μm expression vectors. The resulting plasmids were transformed into DH5α for plasmid storage, and into *Saccharomyces cerevisiae* W303α. Transformation into *Saccharomyces cerevisiae* was performed by a standard lithium acetate protocol. The resulting cells were harvested, washed in PBS, then heat-killed at 56°C for 1 hour in PBS. After heat-kill of the cultures, the cells were washed three times in PBS, and EGFR protein expression was confirmed in each of the compositions by Western blot using EGFR antibody (SCBT, data not shown).

[00169] To prepare the tumor cell lines used in these studies, the 9L gliosarcoma, a malignant glioma cell line syngeneic to the Fisher 344 rat (Charles River Laboratories), was cultured in RPMI-1640 medium supplemented with 10%FBS(cRPMI-10), 50Um2-mercaptoethanil and 2mM glutamine. The cells were grown in a humidified incubator at

37°C with a 5% CO2 air atmosphere. Full-length human or rat cDNA encoding EGFR were inserted into mammalian cell expression vector PCI-neo (Promage). Each plasmid was transfected into separate rat 9L glioma cell lines by effectine transfection reagent (Qiagen), and clones were selected by G418. Verification of EGFR expression was performed by flow cytometry with PE-anti-EGFR antibody (SCBT, SC-120PE) and western blot. The stable cell lines were propagated in cPRMI-10 containing 1mg/ml G418 sulfate. The cloned 9L-E cell line (EGFR-9L) was subsequently sorted for cells that express high, intermediate or low levels of hEGFR. The 9L-E cells therefore possess a human antigen included in various yeast immunotherapy products described herein, and has provided an appropriate surrogate model for human gliomas that exhibit altered expression of EGFR in the malignant cells. 9L cell lines expressing the rat EGFR protein are similarly useful in experiments utilizing the yeast-based immunotherapy composition that expresses rat EGFR (see below), and provides an excellent model of glioblastoma in the rat.

[00170] In a previous experiment described in PCT Publication No. WO 2004/058157 and U.S. Patent No. 7,465,454, a yeast-based immunotherapy composition expressing the human cancer antigen, EGFR, denoted EGFR-tm-VAX, which is GI-3001, was used in the rat glioma model. The goal of the studies was to demonstrate that the yeast-based delivery vehicle triggered protective immunity against challenge with a lethal dose of the 9L-E glioma cells (expressing human EGFR) implanted intracranially into rats. The transfected 9L-E cells formed tumors intracranially in rats (data not shown). This previously published experiment tested whether the yeast-based immunotherapeutic GI-3001 would provide immunotherapeutic protection (prophylactic) against an *intracranial* tumor challenge. Animals (8 animals per group) were immunized with ~20 million yeast cells expressing hEGFR (GI-3001, denoted EGFR-vax) by the intranasal (i.n., Fig. 3, squares) or subcutaneous (s.c., Fig. 3, triangles) route on days 0, 7, 21. Controls included mice immunized with yeast alone (Fig. 3, diamonds). Immunized animals were challenged by intracranial administration of 1,250 cells of the untransfected parent 9L rat glioma (Fig. 3, asterisk) or 9L expressing hEGFR (all other groups). Rat body weights were monitored daily, where loss of body weight was indicative of impending animal mortality.

[00171] The results, shown in Fig. 3, demonstrated that approximately 40-50% of the animals immunized with GI-3001 (EGFR-vax) yeast were completely protected against lethal intracranial tumor challenge with the rat 9L glioma expressing the human EGFR

tumor antigen. None of the animals rejected the growth of parent tumors that lacked the tumor antigen (*i.e.*, the vaccine induces antigen-specific immunity). In addition, the remaining GI-3001-immunized animals that succumbed to the lethal challenge still demonstrated extended survival time as compared to control animals. Therefore, a yeast-based immunotherapy composition expressing a relevant human cancer antigen has been shown to significantly increase the survival and reduce tumor burden *in vivo*.

[00172] In a second experiment, which was not previously published in PCT Publication No. WO 2004/058157 and U.S. Patent No. 7,465,454, yeast expressing the rat EGFR protein (GI-3005) or expressing the human EGFR protein (GI-3006), as compared to non-specific yeast (GI-1001), were evaluated for their ability to target tumors overexpressing rat EGFR proteins (results shown in Fig. 4). Doses were administered IN at days -35, -28, -14 and -7 prior to tumor challenge, then additional immunizations were given on days 7, 14, 21 and 56 after intracranial implantation of 5,000 9L glioma cells overexpressing rat EGFR protein. The Kaplan-Meier plot shown in Fig. 4 demonstrates that immunization with yeast expressing rat EGFR (GI-3005) provided significant survival benefit compared to non-specific yeast (GI-1001), and trended to improved benefit compared to immunization with yeast expressing the human EGFR (GI-3006). These results demonstrate that cell-mediated immune responses against an *endogenous self-antigen* were amplified by yeast-based immunotherapy. Furthermore, immunization with the homologous self antigen (rat EGFR) works as well or better than immunization with a heterologous (xenogeneic) self-antigen (human EGFR).

[00173] In one additional experiment, GI-3010 was administered in the rat glioma model as described above using intranasal and oral administration. Briefly, rats were dosed intranasally or orally with GI-3010 (expressing human EGFR) on Day -27, Day -21, Day -14, and then challenged with human EGFR 9L tumor cells (5,000 cells), followed by additional dosing with GI-3010 at Day 7 and Day 14. As shown in Fig. 5A, mice receiving GI-3010 demonstrated a survival benefit as compared to mice receiving a placebo. As shown in Fig. 5B, cells effector cells from mice immunized with GI-3010 i.n. also showed cytotoxic T cell (CTL) activity as demonstrated by the ability of effector cells to lyse ^{51}Cr-labeled human EGFR 9L targets.

[00174] Taken together, these data show that yeast-based immunotherapeutic compositions elicit antigen-specific immune responses against a cancer target in a rat model of glioblastoma, are capable of eliciting immune responses against an endogenous

self antigen as well as a heterologous self antigen, and protect mice from tumors by increasing and/or prolonging survival of animals in the face of tumor challenge.

Example 4

[00175] The following example demonstrates the use of yeast-based immunotherapy comprising a human cancer antigen, in combination with arginine therapy, to treat a brain tumor *in vivo*.

[00176] With respect to the treated animals in the experiments described in Example 3 above that still succumbed to tumor, the experimental data provided herein and shown previously in the rat glioma model indicate that tumor-associated immunosuppression by MDSC-mediated mechanisms ultimately contributed to their demise. Accordingly, the following experiment describes the targeting of MDSC-derived immunosuppression to augment endogenous anti-tumor immunity and increase efficacy of standard immunotherapy using tumor vaccination with yeast-based immunotherapy. While the experiments in Examples 1 and 2 show that targeting MDSC in isolation will provide therapeutic benefit for improving cellular immune function in individuals with MDSC-mediated immunosuppression, a combinatorial approach targeting MDSC with concurrent tumor-specific immune stimulation provides a more robust and lasting immunological response.

[00177] ***Combination Yeast-Based Immunotherapy and Arginine Therapy:*** Although rescue of global cellular immune function using arginine therapy alone is expected to provide a detectable anti-tumor effect, concurrent stimulation of tumor-specific immunity is desirable, if not required, for the most effective use of the present invention. Targeting MDSC-derived immunosuppression in the setting of concurrent immunotherapy provides an optimal combinatorial approach to facilitate immune-mediated tumor clearance and improve survival.

[00178] *Vaccination strategy and efficacy of targeting ArgI:* Rats are intracerebrally inoculated with EGFR-9L tumors as described in Example 2 and Example 3 above. EGFR-9L tumors expressing rat EGFR, and the corresponding yeast-based immunotherapy composition (GI-3005) is administered. The immunization strategy consists of immunization subcutaneously with yeast-EGFR immunotherapy compositions (GI-3005), at 20 million yeast per vaccination, administered weekly for at least 4-5 doses, followed by additional monthly doses. Specifically, animals receive an initial vaccination at the time of tumor implantation (day 0), and additional immunizations are performed at

weekly intervals until day 28, at which time surviving animals will be switched to a monthly maintenance immunization. The dose of yeast is administered to a single site on the animal, or is equally divided among 2-4 different sites at each dosing. In addition, additional dose cohorts are added to the study in some experiments. Groups of study animals receive treatment with oral L-arginine or i.p. NOHA or nor-NOHA (arginase inhibitors) as described in Example 2. As controls, sham-vaccinated animals are used for assessment of baseline tumor immune responses and overall survival.

[00179] Animals are sacrificed at the time points outlined in Example 2 and tissues are prepared for phenotypic and functional analysis of T cells within the various compartments as described in Example 2. Arginine therapy, either by L-arginine supplementation and/or by treatment with arginase I inhibitor, is expected to result in increased vaccine-mediated immune infiltration of tumors (as compared to sham vaccinated animals) and increased survival, as compared to animals not treated with arginine therapy and as compared to negative control animals. Since prior experiments in this model using yeast-EGFR immunotherapy alone indicated that more than 50% of animals die by day 30, an increase in overall survival, prolonged survival, and/or complete cure during the course of the experiment in a greater percentage of vaccinated animals through targeting of MDSC-mediated immunosuppression is considered to be a positive result. Overall, augmentation of endogenous cellular immune function and increased efficacy of yeast-based immunotherapy in tumor-bearing rats treated with oral Arg or i.p. NOHA or nor-NOHA is expected.

[00180] Statistical analysis: Analysis of survival data utilizes Kaplan-Meier and Wilcoxon rank-sum testing. Comparative analysis of continuous variables at the various time points utilizes repeated measures ANOVA.

[00181] These experiments are designed to demonstrate that the combination of arginine therapy and yeast-based immunotherapy inhibits the functional immunosuppressive activity of CD11b+/His48+ MDSC generated in some cancers and other diseases, and improves immunological and therapeutic outcomes elicited by each therapy.

Example 5

[00182] The following experiment describes a phase 0 clinical study showing that MDSC-derived T cell dysfunction, mediated by arginine depletion, can be reversed in GBM patients through the use of oral arginine supplementation.

[00183] While prior clinical studies have studied arginine supplementation as a component of immunonutrition in patients undergoing major surgery or under intensive care, no previous clinical trial has specifically focused upon the use of oral arginine supplementation to reverse MDSC-derived immunosuppression in cancer patients.

[00184] The primary objective of this study is to demonstrate a significant increase in mitogen-induced T cell interferon gamma (IFN-γ) production between baseline pre-operative samples and samples taken at the time of surgery in GBM patients treated with oral arginine supplementation. Secondary objectives include generation of preliminary data regarding safety of oral arginine supplementation in patients with GBM, normalization of CD4:CD8 ratios and CD3ζ expression levels within the circulating T cell compartment, restoration of T cell proliferative responses *in vitro*, detection of increased tumor immune infiltration at the time of surgery, and evaluation of Th1/Th2 bias, and representation of regulatory T cells within tumor-infiltrating lymphocytes. The frequency of MDSC and serum ArgI within both experimental groups is also compared.

[00185] *Patient population:* The study is open to all patients over the age of 18, regardless of gender or ethnic background, with newly diagnosed GBM on brain imaging. Subjects are additionally eligible for study participation provided they meet the following inclusion criteria: (1) imaging consistent with glioblastoma multiforme without clinical indication for primary CNS lymphoma or abscess, as determined by the treating physician; (2) patient must be planned to proceed to definitive surgery intended for tumor resection, rather than needle biopsy, within a reasonable time frame from initial evaluation (7-14 days); (3) patient must be neurologically stable, allowing for reasonable time frame between initial evaluation and subsequent surgical procedure (7-14 days); (4) Karnofsky performance status greater than 80 and ECOG of 1 or 0; (5) at the time of initial evaluation the patient must be on a stable dose of steroid medication; (6) patient must have laboratory values, as determined by institutional controls, within the following parameters: (a) white blood cell count above lowest level for normal range; (b) renal function within normal limits (Creatinine, BUN); (c) liver function within normal limits (AST/ALT, Total bilirubin, alkaline phosphatase); (7) written informed consent is obtained prior to initiation of study procedures. Multiple exclusion criteria are also applied (not shown here).

[00186] *Treatment Plan:* Following patient accrual and randomization to either oral arginine supplementation or placebo, patients are provided with the study drug and instructed to start treatment on day 7 prior to the planned surgical procedure. Patients

randomized to the arginine arm will take a total of 24 mg L-arginine tablets divided into four equivalent daily doses. This dose was selected based upon multiple prior human clinical trials demonstrating efficacy and safety. Patients randomized to placebo take tablets of identical size and appearance on a similar dosing schedule. Patients in both arms are instructed to continue the daily regimen through the day of their planned surgical procedure. Following surgery, patients are restarted on post-operative day 1 or the soonest possible post-operative day upon which they are able to tolerate oral intake. Patients continue the drug until the first post-operative visit, which is typically 7-10 days following the surgical procedure.

[00187] *Justification for Randomization and Placebo:* Prior study has suggested that the placebo effect may exert significant influence over immune responses. As preliminary data provided a single time point regarding baseline immune function at the time of surgery in patients with GBM, the placebo arm is important to provide detection of a positive immunological effect from participation in the trial.

[00188] *Clinical Monitoring:* Patients initially undergo baseline clinical laboratory blood testing to confirm eligibility for inclusion criteria. At the time of the scheduled surgery and at the first post-operative visit, the research nurse acquires data regarding study compliance, including details involving any missed doses and a review of any adverse effects. Patients undergo physical examination, including vital signs and update of Karnofsky Performance status, and a blood draw is performed allowing for acquisition of clinical labs as well as blood for laboratory studies.

[00189] *T cell functional analysis:* Peripheral blood samples taken at the aforementioned time points are transported to the research lab for processing and analysis. PBMCs are purified using Ficoll density centrifugation (Sigma-Aldrich). Triplicate samples are subject to PHA stimulation (Sigma) as describe in earlier examples. At the appropriate time points, media is harvested and used for IFN-γ ELISA analysis (R&D Systems). All research staff are blinded to the study assignment of each patient. Absolute values of IFN-γ production are averaged across patients in each group and used for comparative analysis. As the primary outcome measure for the study, it is expected that arginine supplementation will result in a 25% restoration of IFN-γ production over baseline in arginine-treated patients without significant change in the placebo group. Additional populations of PBMC are stained with CFSE (Invitrogen) per standard protocols and stimulated with PHA as above. At the aforementioned time points, cells are

harvested for comparative flow cytometric analysis to determine the ratio of proliferating to resting CD3+ T cells within stimulated samples and establish a proliferation index (PI). Averaged PI values for each study group are compiled. It is expected that T cell proliferation in Arg-treated patients will exceed placebo-treated patients in significant fashion.

[00190] *Peripheral T cell phenotypic analysis:* PBMC obtained at each study time point are stained for flow cytometric analysis per standard protocols. A subset of samples are fixed and permeabilized for intracellular staining. Flourophor-conjugated antibodies against CD3, CD4, and CD8 (BD Biosciences) are used to determine restoration of CD4:CD8 ratios in treated patients. Intracellular staining for CD3ζ (BD Biosciences) is also performed to evaluate for Arg-mediated rescue of functional TCR expression in treated patients.

[00191] *Flow cytometric analysis of tumor-infiltrating lymphocytes:* Tumor specimens are taken at the time of surgery and transported directly to the research lab in cold media. Specimens are reduced to single-cell suspension, stained, and used for flow cytometric analysis. Relative proportions of T cell populations are identified by staining for CD3+, CD4+, and CD8+ cells. Additional samples are fixed and permeabilized for intracellular Ki-67 staining (a surrogate marker for proliferation).

[00192] Immunohistochemical *evaluation of tumor-infiltrating T cells:* Tumor sections are stained for CD3, CD4, and CD8 to evaluate T cell frequency and intratumoral penetration. Slides are reviewed and scored, focusing on areas of infiltrative (non-necrotic) tumor, using a four-tiered system: 1) No infiltrate, 2) Minimal perivascular infiltrate, 3) Perivascular infiltrate with extension into tumor parenchyma, and 4) Significant infiltrate into tumor parenchyma.

[00193] Evaluation *of MDSC frequency and serum ArgI levels:* PBMCs are assessed using flow cytometry to detect frequency of MDSC in each study group. Serum ArgI is measured using ELISA (Abnova).

[00194] *Sample size considerations:* Preliminary data shows that the mean baseline IFN-γ production by GBM PBMC at 48 hr of *in vitro* mitogenic stimulation is 177 ug with a standard deviation (SD) of 15.1 in 10 GBM patients. The sample size calculation is based on the changes (at surgery minus baseline) of IFN-γ on (natural) log scale as the logarithmic transformation makes the variable distribution more symmetric and stabilizes variance. It is assumed that the mean baseline IFN-γ is 180 ug for both arginine-treated

and placebo groups, and an increase of 25% (IFN-γ=225) and 10% (IFN-γ=189) will be observed at the time of surgery in arginine-treated and placebo groups, respectively. It is also assumed, conservatively, that the variance of IFN-γ at surgery in the treated group is twice the variance at baseline.

[00195] To be conservative, it is further assumed that the variance of the changes equal to the summation of baseline variance and variance at the time of surgery for both groups. Thus, the calculated mean change and the SD are 0.22 (0.124) and 0.09 (0.113) (log scale), respectively, for the treated and placebo groups. Using a two sample t-test with 13 patients in each group this treatment difference would be detected with 85% power at the 0.05 significance level (one-sided).

[00196] *Primary efficacy* endpoint: A two-sample t-test is used to compare the change in IFN-γ production (at surgery minus baseline) between the two groups on the natural log scale. A p-value is reported along with the change and its 95% confidence interval on the original scale for both groups.

[00197] *Secondary* endpoints*:* The analyses for the secondary endpoints is hypothesis–generating and descriptive in manner. For continuous variables, potential differences in the change (at surgery minus baseline) is evaluated between the two groups using a t-test or Wilcoxon rank sum test. In addition, repeated measures ANOVA are used to assess whether the treatment effect on IFN-γ induction is maintained at the first follow-up visit. All patients except those who withdraw from the trial due to intolerance to arginine are included for efficacy analyses.

Example 6

[00198] The following example describes a Phase 1 clinical trial in humans.

[00199] Following positive results of the P0 clinical trial described above, a phase 1 clinical trial is initiated in humans to evaluate the combination of arginine therapy and yeast-based immunotherapy in cancer.

[00200] An open-label, dose-escalation phase 1 clinical trial is run using a yeast-EGFR immunotherapy composition known as GI-3010 described in Example 3. 12-24 subjects with a EGFR-positive brain tumor (EGFRviii included) classified as glioblastoma multiforme and meeting the criteria described for the P0 trial described in Example 4 above are treated using standard of care (SOC) therapy for GBM with one cohort receiving supplemental arginine and yeast-based immunotherapy and the other cohort receiving SOC alone plus placebo. Following patient accrual and randomization to either

oral arginine supplementation/yeast-based immunotherapy or placebo, patients are provided with oral arginine and instructed to start treatment on day 7 prior to the planned surgical procedure. Patients randomized to the arginine/immunotherapy arm take a total of 24 mg L-arginine tablets divided into four equivalent daily doses. Patients randomized to placebo take tablets of identical size and appearance on a similar dosing schedule. Patients in both arms are instructed to continue the daily regimen through the day of their planned surgical procedure. Following surgery, patients are restarted on arginine therapy post-operative day 1 or the soonest possible post-operative day upon which they are able to tolerate oral intake. They then continue arginine until the conclusion of the study.

[00201] Patients randomized into the arginine/immunotherapy arm are also administered the yeast-EGFR 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-EGFR immunotherapy is administered at 2 week intervals beginning either prior to surgery, or beginning 1-2 weeks after surgery for 3 months, and then monthly.

[00202] The results monitor safety as a primary endpoint, and as secondary endpoints, immunology endpoints as described above for the P0 trial, as well as clinical activity.

[00203] Combination arginine and yeast-based immunotherapy is expected to be safe and well-tolerated with no significant toxicities. In addition, combination therapy is expected to produce treatment-emergent EGFR-specific T cell responses or an improvement in pre-existing EGFR-specific baseline T cell responses in a statistically significant number of patients. Some patients are also expected to have stabilized disease and increased survival.

[00204] 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 method to treat a disease associated with myeloid-derived suppressor cell (MDSC)-mediated or arginase-mediated immunosuppression, comprising administering to an individual with the disease a yeast-based immunotherapy composition and arginine therapy.

2. The method of Claim 1, wherein the arginine therapy comprises administering an oral arginine supplement to the individual.

3. The method of Claim 2, wherein the arginine supplement is L-arginine.

4. The method of Claim 2, wherein the L-arginine is administered daily.

5. The method of Claim 2, wherein the arginine supplement is administered as a powder, a tablet, or a capsule.

6. The method of Claim 1, wherein the arginine therapy comprises administration of an arginase inhibitor to the individual.

7. The method of Claim 6, wherein the arginase inhibitor is selected from the group consisting of: nor-NOHA (N(omega)-Hydroxy-nor-L-arginine) and NOHA (N^G-Hydroxy-L-arginine).

8. The method of any one of Claims 1 to 7, wherein the yeast-based immunotherapy composition comprises a yeast vehicle that recombinantly expresses an antigen associated with the disease.

9. The method of Claim 8, wherein the yeast vehicle is a whole, heat-killed yeast.

10. The method of Claim 8, wherein the yeast vehicle is from *Saccharomyces.*

11. The method of Claim 8, wherein the yeast-based immunotherapy composition is administered weekly for 1-5 weeks, followed by monthly administration for 3-24 months.

12. The method of Claim 8, wherein the disease is cancer.

13. The method of Claim 12, wherein the antigen is an epidermal growth factor receptor (EGFR) antigen.

14. The method of Claim 12, wherein the antigen is a cancer antigen expressed by a brain cancer.

15. The method of Claim 14, wherein the brain cancer is glioblastoma multiforme.

16. The method of Claim 14, wherein the cancer antigen is selected from the group consisting of: epidermal growth factor receptor (EGFR) including EGFRvIII, mutated Ras (having a mutation at position 12, 13, 59 and/or 61), mitogen-activated protein kinase (MAPK), interleukin-13 receptor-α2 (IL-13Rα2), gp100, TRP-2, MAGE-A3, MAGE-1, Her-2/neu, EphA2, survivin, Wilm's Tumor 1 (WT1), Sry-Related High-Mobility Group Box-2 (SOX2), SOX11, AIM2, and Squamous Cell Carcinoma Antigen Recognized by T Cells 1 (SART1).

17. The method of Claim 12, wherein the antigen is a cancer antigen expressed by a lung cancer.

18. The method of Claim 17, wherein the antigen is selected from the group consisting of: mutated Ras (mutations at positions 12, 13, 59 and/or 61), carcinoembryonic antigen (CEA), mucin-1 (MUC1), Brachyury, EGFR, Her2/neu, NY-ESO-1, and MAGE-A3.

19. The method of Claim 12, wherein the antigen is a cancer antigen expressed by a renal cell carcinoma.

20. The method of Claim 19, wherein the antigen is selected from the group consisting of: CEA, MUC1, Brachyury, mutated Ras, EGFR, multidrug resistance-associated protein 3, polycomb group protein enhancer of zeste homologue 2, Her2/neu, von Hippel-Lindau (VHL) protein, kidney cancer antigen 1 (KCAG1), TRP-2, FGF-5, and renal cell carcinoma-associated antigen G250.

21. The method of Claim 12, wherein the antigen is a cancer antigen expressed by a breast cancer.

22. The method of Claim 21, wherein the antigen is selected from the group consisting of: CEA, MUC1, Brachyury, mutated Ras, EGFR, TWIST, hTERT, Her2/neu, NY-BR-1, EphA2, CTAG1, carbonic anhydrase IX (CA9), MAGE-A3, MAGE-1, BAGE-1, HERV-K and HERV-H.

23. The method of Claim 12, wherein the antigen is a cancer antigen expressed by a pancreas cancer.

24. The method of Claim 23, wherein the antigen is selected from the group consisting of: CEA, MUC1, Brachyury, mutated Ras, EGFR, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, LAGE-1, NY-ESO-1, SCP-1, SSX-2, SSX-4, HERV-K-MEL and HERV-H.

25. The method of Claim 12, wherein the antigen is a cancer antigen expressed by a colorectal cancer.

26. The method of Claim 25, wherein the antigen is selected from the group consisting of: CEA, MUC1, Brachyury, mutated Ras, EGFR, CTAG1, MAGE-A3, MAGE-1, BAGE1, GCC, Her2/neu, carbonic anhydrase IX (CA9), HERV-K and HERV-H.

27. The method of Claim 1, wherein the method comprises the steps of:

a) Administering the arginine therapy as a monotherapy for a period of from 1 to 30 days prior to administering the yeast-based composition;

b) After step (a), administering the yeast-based composition weekly for five weeks followed by 3 to 24 monthly doses, while continuing to administer the arginine therapy daily;

c) After step (b), administering the arginine therapy as a monotherapy for an additional 1 to 12 months or longer.

28. A kit comprising a yeast-based immunotherapy composition and an arginine supplement or an inhibitor of arginase.

29. The kit of Claim 28, wherein the arginine supplement is L-arginine.

30. The kit of Claim 28, wherein the yeast-based immunotherapy composition comprises a yeast vehicle that recombinantly expresses an antigen associated with the disease.

31. The kit of Claim 31, wherein the yeast vehicle is a whole, heat-killed yeast.

32. The kit of Claim 31, wherein the antigen is a cancer antigen.

33. The kit of Claim 31, wherein the antigen is a cancer antigen from a brain cancer, a lung cancer, a renal cell carcinoma, a breast cancer, a pancreas cancer, or a colorectal cancer.

34. Use of a yeast-based immunotherapy composition and arginine therapy in the preparation of a medicament for the treatment of a disease.

35. Use of a yeast-based immunotherapy composition and arginine therapy to treat cancer.

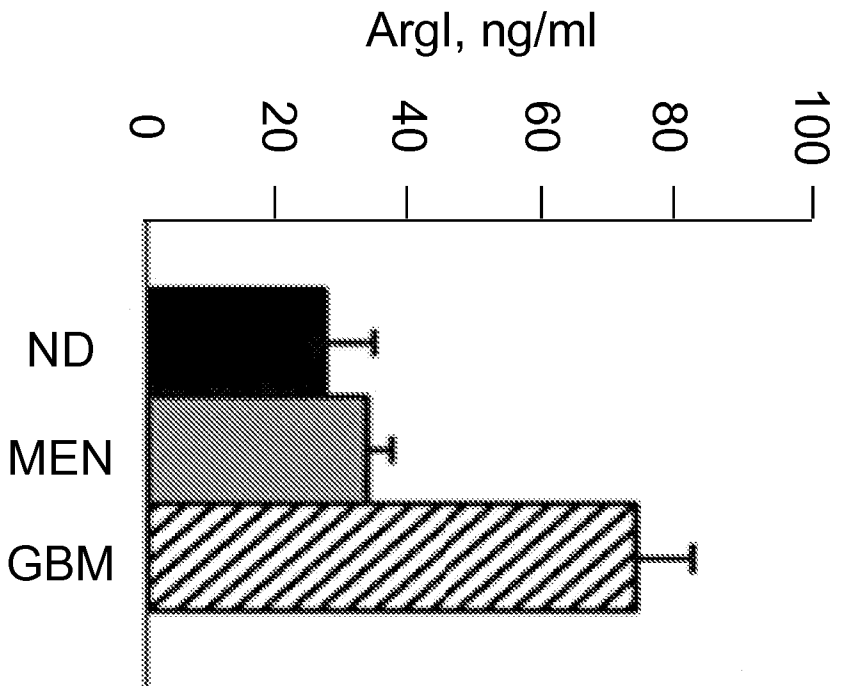


FIG. 1

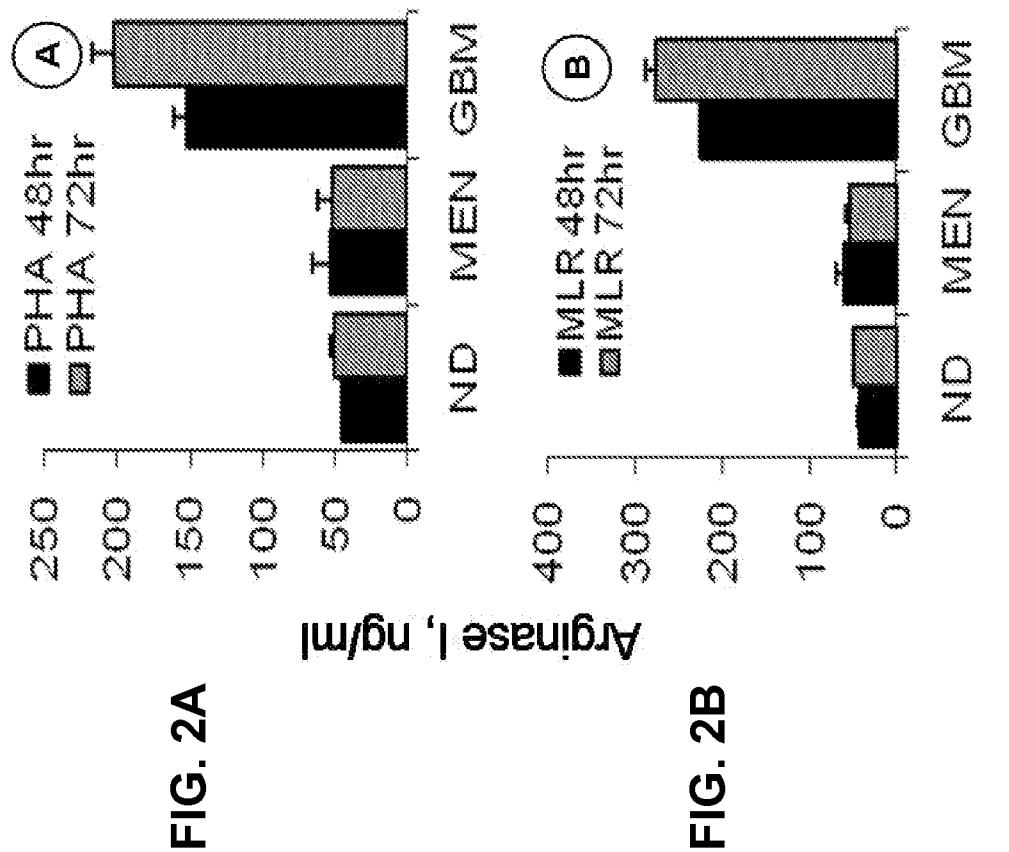


FIG. 2A

FIG. 2B

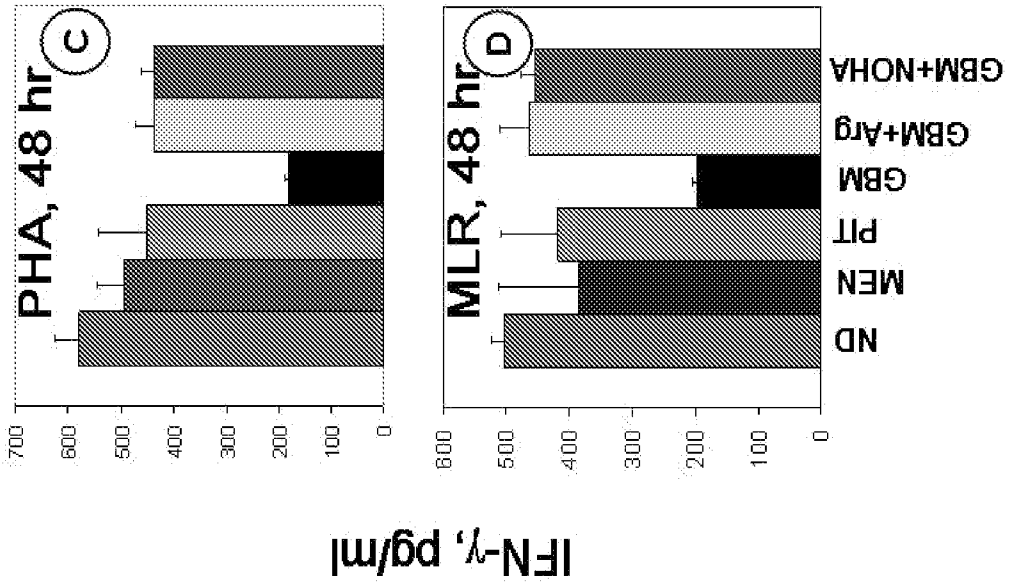


FIG. 2C

FIG. 2D

FIG. 3

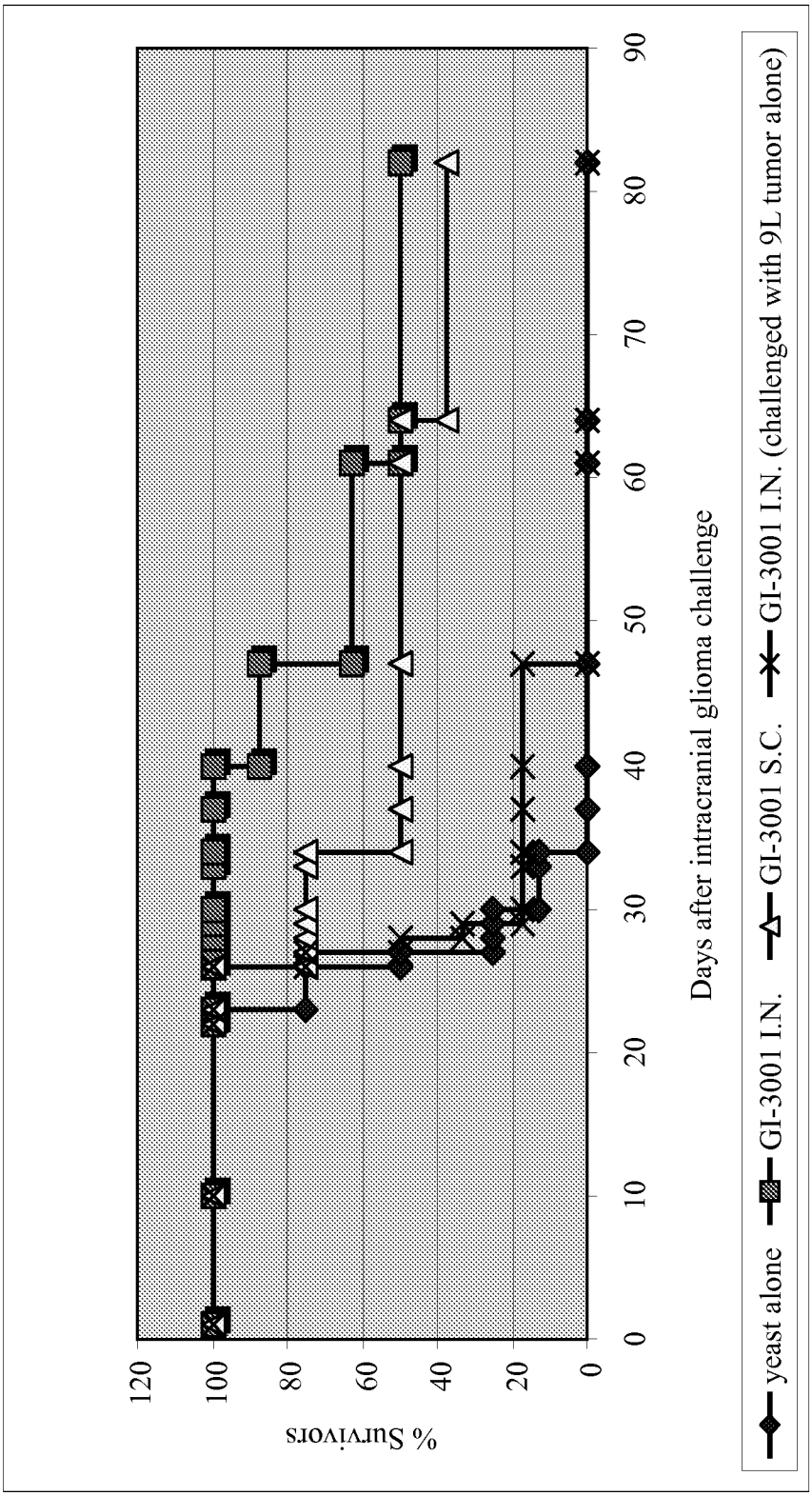

% Survivors
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Days after intracranial glioma challenge
yeast alone
GI-3001 I.N.
GI-3001 S.C.
GI-3001 I.N. (challenged with 9L tumor alone)

FIG. 4

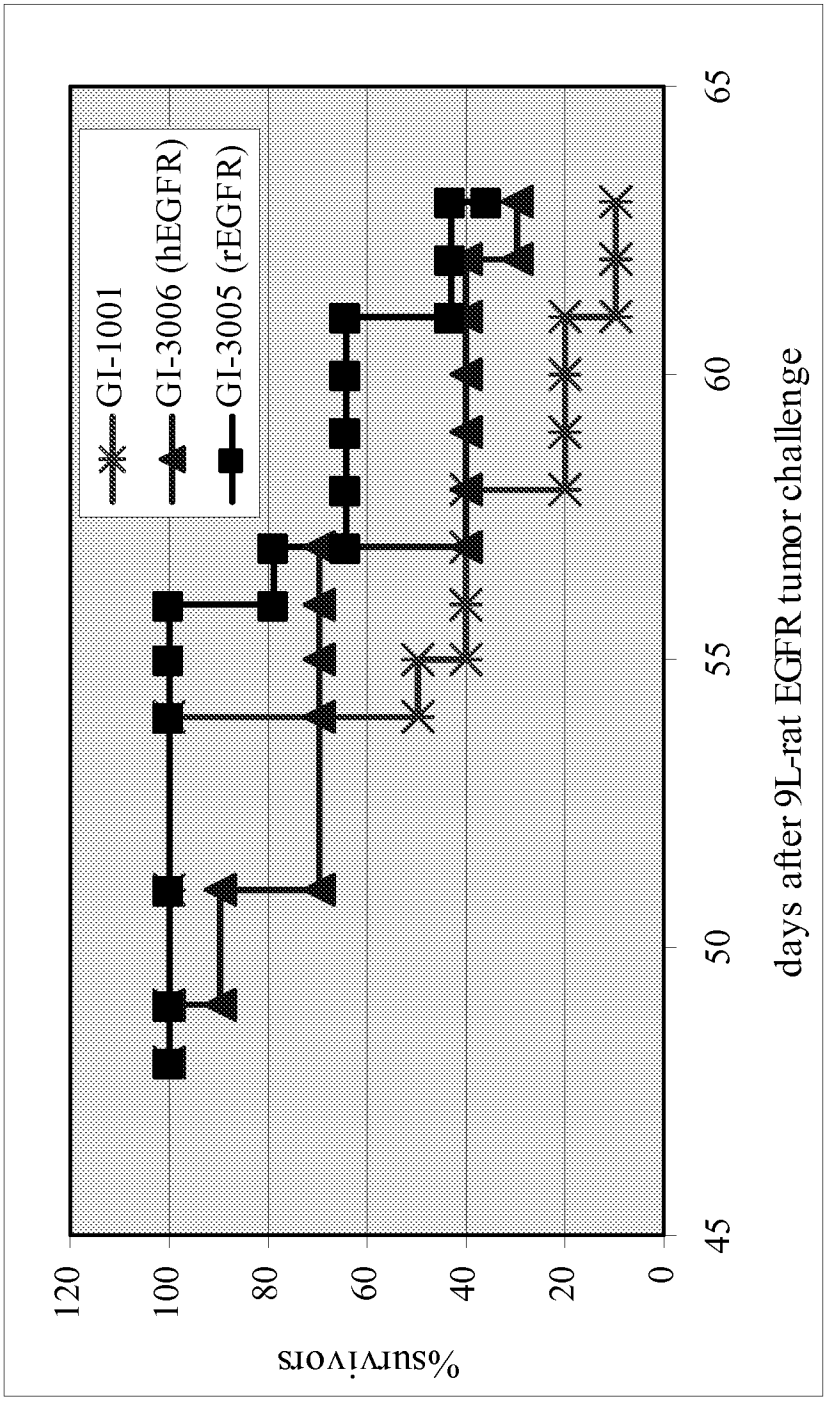

GI-1001
GI-3006 (hEGFR)
GI-3005 (rEGFR)
%survivors
120
100
80
60
40
20
0
45
50
55
60
65
days after 9L-rat EGFR tumor challenge

FIG. 5A

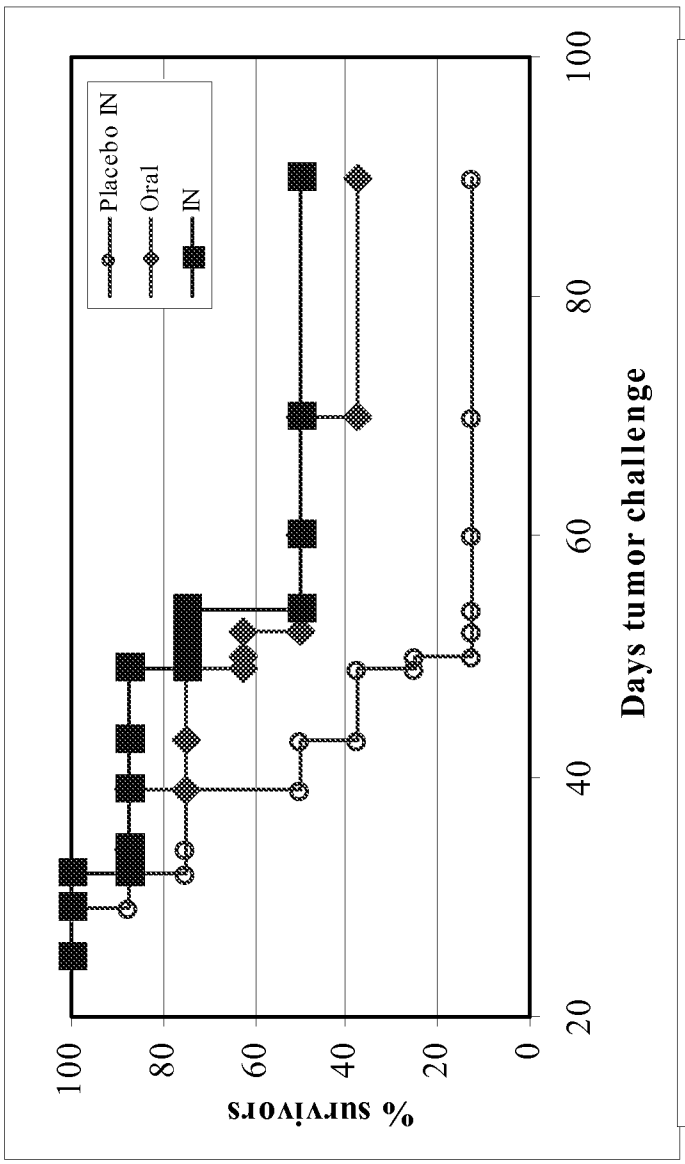

Placebo IN
Oral
IN
% survivors
Days tumor challenge
0
20
40
60
80
100
20
40
60
80
100

FIG. 5B

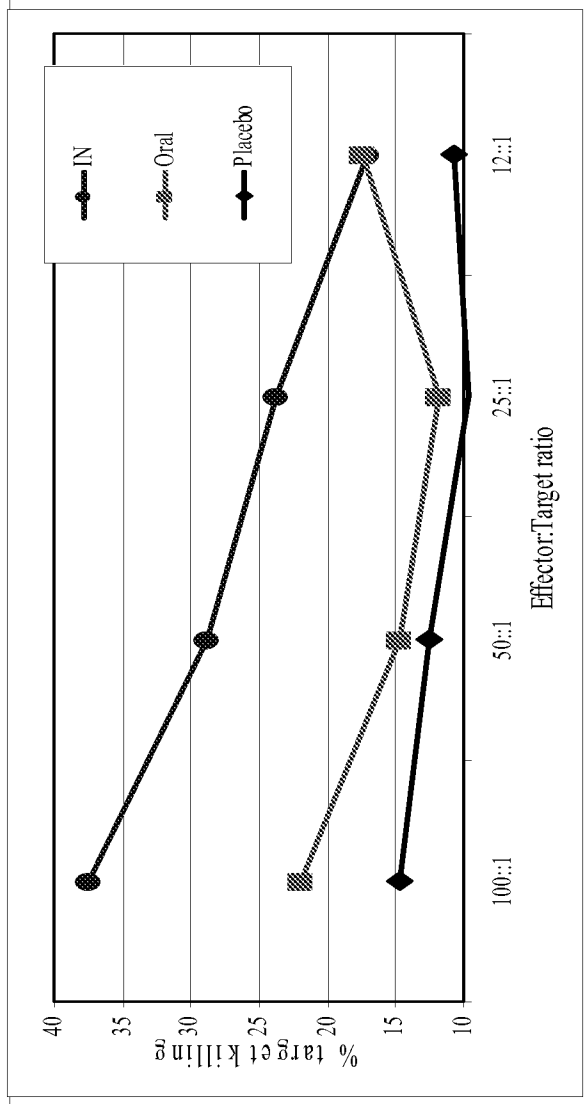
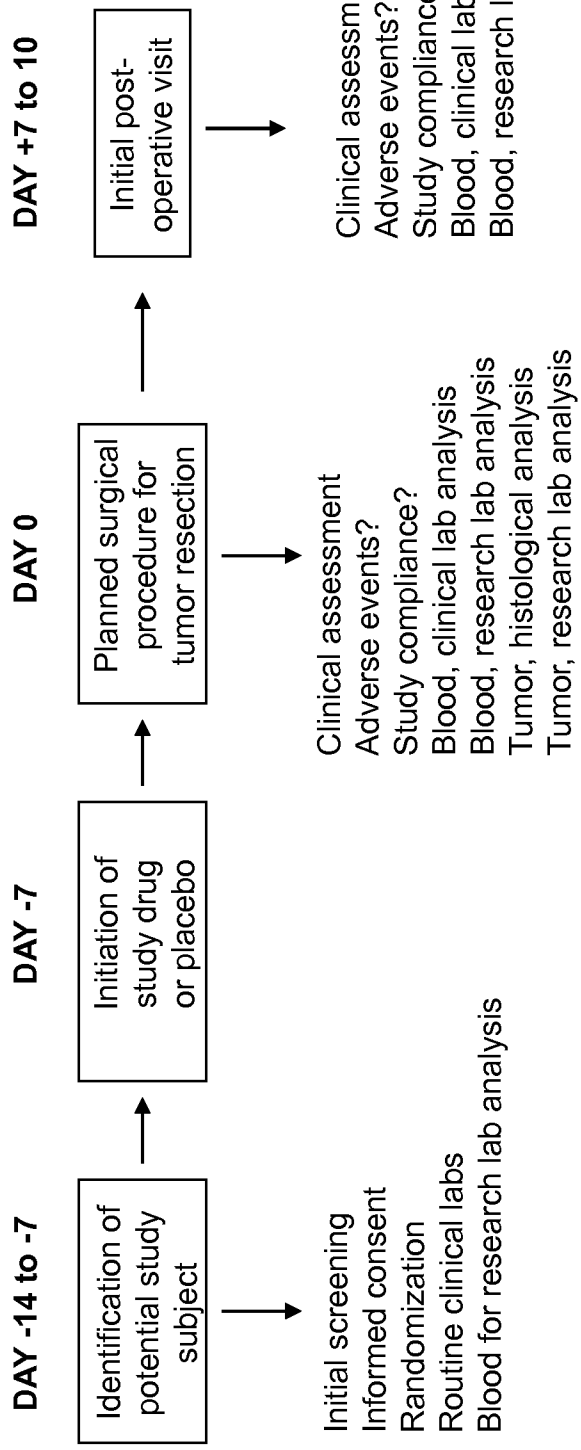

IN
Oral
Placebo
% target killing
Effector:Target ratio
100::1
50::1
25::1
12::1
10
15
20
25
30
35
40

FIG. 6


DAY -14 to -7
Identification of potential study subject
Initial screening
Informed consent
Randomization
Routine clinical labs
Blood for research lab analysis
DAY -7
Initiation of study drug or placebo
DAY 0
Planned surgical procedure for tumor resection
Clinical assessment
Adverse events?
Study compliance?
Blood, clinical lab analysis
Blood, research lab analysis
Tumor, histological analysis
Tumor, research lab analysis
DAY +7 to 10
Initial post-operative visit
Clinical assessment
Adverse events?
Study compliance?
Blood, clinical lab analysis
Blood, research lab analysis