MODIFIED RECOMBINANT VACCINIA VIRUSES AND OTHER MICROORGANISMS, USES THEREOF

RELATED APPLICATIONS

Benefit of priority is claimed to each of EP 03 013 826.7, filed 18 June 2003, entitled "Recombinant vaccinia viruses useful as tumor-specific delivery vehicle for cancer gene therapy and vaccination; " EP 03 018 478.2, filed 14 August 2003, entitled "Method for the production of a polypeptide, RNA or other compound in tumor tissue;" and EP 03 024 283.8, filed 22 October 2003, entitled "Use of a Microorganism or Cell to Induce Autoimmunization of an Organism Against a Tumor." Where permitted, the subject matter of each of these applications is incorporated by reference in its entirety.

This application also is related U.S. application Serial No. 10/872,156, filed on the same day herewith. This application also is related to U.S. Application filed June 10, 2004 Serial No. 10/866,606, entitled "Light emitting microorganisms and cells for diagnosis and therapy of tumors," which is a continuation of U.S. Application Serial No. 10/189,918, filed July 3, 2002; U.S. Application filed May 19, 2004 Serial No. 10/849,664 entitled, "Light emitting microorganisms and cells for diagnosis and therapy of diseases associated with wounded or inflamed tissue" which is a continuation of U.S. Application Serial No. 10/163,763, filed June 5, 2003; International PCT Application WO 03/014380, filed July 31, 2002, entitled "Microoroganisms and Cells for Diagnosis and Therapy of Tumors; " PCT Application WO 03/104485, filed June 5, 2003, entitled, "Light Emitting Microorganisms and Cells for Diagnosis and Therapy of Diseases Associated with Wounded or Inflamed tissue;" EP Application No. 01 118 417.3, filed July 31, 2001, entitled "Light-emitting microorganisms and cells for tumour diagnosis/therapy;" EP Application No. 01 125 911.6, filed October 30, 2001, entitled "Light emitting microorganisms and cells for diagnosis and therapy of tumors;" EP Application No. 02 0794 632.6, filed January 28, 2004, entitled "Microorganisms and Cells for Diagnosis and Therapy of Tumors;" and EP Application No. 02 012 552.2, filed June 5, 2002, entitled "Light Emitting

Microorganisms and Cells for Diagnosis and Therapy of Diseases associated with wounded or inflamed tissue." Where permitted, the subject matter of each of these applications is incorporated by reference in its entirety.

FIELD OF THE INVENTION

Vaccines that contain attenuated or modified microorganisms, including microbes and cells, and methods for preparing the microorganisms and vaccines are provided. In particular, modified bacteria, eukaryotic cells and viruses are provided and methods of use thereof for treatment of proliferative and inflammatory disorders and for production of products in tumors are provided.

BACKGROUND

In the late 19th century, a variety of attempts were made to treat cancer patients with microorganisms. One surgeon, William Coley, administered live Streptococcus pyogenes to patients with tumors with limited success. In the early 20th century, scientists documented vaccinia viral oncolysis in mice, which led to administration of several live viruses to patients with tumors from the 1940s through the 1960s. These forays into this avenue of cancer treatment were not successful.

Since that time, a variety of genetically engineered viruses have been tested for treatment of cancers. In one study, for example, nude mice bearing nonmetastatic colon adenocarcinoma cells were systemically injected with a WR strain of vaccinia virus modified by having a vaccinia growth factor deletion and an enhanced green fluorescence protein inserted into the thymidine kinase locus. The virus was observed to have antitumor effect, including one complete response, despite a lack of exogenous therapeutic genes in the modified virus (McCart *et al.* (2001) *Cancer Res 1*:8751-8757). In another study, vaccinia melanoma oncolysate (VMO) was injected into sites near melanoma positive lymph nodes in a Phase III clinical trial of melanoma patients. As a control, New York City Board of Health strain vaccinia virus (VV) was administered to melanoma patients. The melanoma patients treated with VMO had a survival rate better than that for untreated patients, but similar to patients treated with the VV control (Kim *et al.* (2001) *Surgical Oncol 10*:53-59).

Other studies have demonstrated limited success with this approach. This therapy is not completely effective, particularly for systemically delivered viruses or bacteria. Limitations on the control of microbial vehicle function in vivo result in ineffective therapeutic results as well as raising safety concerns. It would be desirable to improve this type of therapy or to develop more effective approaches for treatments of neoplastic disease. Therefore, among the objects herein, it is an object to provide therapeutic methods and microorganisms for the treatment of neoplastic and other diseases.

SUMMARY

Provided herein are therapeutic methods and microorganisms, including viruses, bacteria and eukaryotic cells, for uses in the methods for the treatment of neoplastic diseases and other diseases. Diseases for treatment are those in which the targeted tissues and/or cells are immunoprivileged in that they, and often the local environment thereof, somehow escape or are inaccessible to the immune system. Such tissues include tumors and other tissues and cells involved in other proliferative disorders, wounds and other tissues involved in inflammatory responses. The microorganisms, which include bacterial cells, viruses and mammalian cells, are selected or are designed to be non-pathogenic and to preferentially accumulate in the immunoprivileged tissues. The microorgamisms, once in the tissues or cells or vicinity thereof, affect the cell membranes of the cells in such tissues so that they become leaky or lyse, but sufficiently slowly so that the targeted cells and tumors leak enough antigen or other proteins for a time sufficient to elicit an immune response.

The microorganisms are administered by any route, including systemic administration, such as i.v. or using oral or nasal or other delivery systems that direct agents to the lymphatics. In exemplary methods, the microorganisms are used to treat tumors and to prevent recurrence and metastatic spread. Exemplary microorganisms include highly attenuated viruses and bacteria, as well as mammalian cells. The microorganisms are optionally modified to deliver other products, including other therapeutic products to the targeted tissues.

When the microorganisms are administered to a host that contains tumors, the tumors in the host essentially become antigen and protein factories. This can be exploited so that the tumors can be used to produce proteins or other cellular products encoded by or produced by the microorganisms. In addition, the host sera can be harvested to isolate antibodies to products produced by the microorganisms as well as the tumor cells. Hence also provided are methods for producing gene products by administering the microorganisms to an animal, generally a non-human animal, and harvesting the tumors to isolate the product. Also provided are methods for producing antibodies to selected proteins or cell products, such as metabolites or intermediates, by administering a microorganism that expresses or produces the protein or other product to a host, typically a non-human host; and harvesting serum from the host and isolating antibodies that specifically bind to the protein or other product.

Thus provided are methods and microorganisms for elimination of immunoprivileged cells or tissues, particularly tumors. The methods include administration, typically systemic administration, with a microorganism that preferentially accumulates in immunoprivileged cells, such as tumor cells, resulting in leakage proteins and other compounds, such as tumor antigens, resulting in vaccination of the host against non-host proteins and, such as the tumor antigens, providing for elimination of the immunoprivileged cells, such as tumor cells, by the host's immune system. The microorganisms are selected not for their ability to rapidly lyse cells, but rather for the ability to accumulate in immunoprivileged cells, such as tumors, resulting in a leakage of antigens in a sufficient amount and for a sufficient time to elicit an immune response.

Hence provided are uses of microorganisms or cells containing heterologous DNA, polypeptides or RNA to induce autoimmunization of an organism against a tumor. In particular, the microorganisms are selected or designed to accumulate in tumors and to accumulate very little, if at all (to be non-toxic to the host) in non-tumorous cells, tissues or organs, and to in some manner result in the tumor cell lyses or cell membrane disruption such that tumor antigens leak. Exemplary of such

microorganisms are the LIVP-derived vaccinia virus and the bacteria described herein and also mammalian cells modified to target the tumors and to disrupt the cells membrane. The microorganisms can be modified to express heterologous products that mediate or increase the leakage of the tumor cell antigens and/or that are therapeutic, such as anti-tumor compounds.

Also provided are methods for production of antibodies against a tumor by (a) injecting a microorganism or cell containing a DNA sequence encoding a desired polypeptide or RNA into an organism bearing a tumor and (b) isolating antibodies against the tumor.

Provided are attenuated microorganisms that accumulate in immunoprivileged tissues and cells, such as tumor cells, but do not accumulate to toxic levels in non-targeted organs and tissues, and that upon administration to an animal bearing the immunoprivileged tissues and cells, result in autoimmunity, such as by production of anti-tumor (or anti-tumor antigen) antibodies against the immunoprivileged cells or products thereof. The microorganisms are selected or produced to render the immunoprivileged cells leaky, such as by a slow lysis or apoptotic process. The goal is to achieve such leakiness, but to not lyse the cells so rapidly that the host cannot mount an immune response.

Uses of and methods of use of the microorganisms for eliminating immunoprivileged are provided. The microorganisms optionally include reporter genes and/or other heterologous nucleic acids that disrupt genes in the microorganism and can also encode and provide therapeutic products or products, such as RNA, including RNAi, that alter gene and/or protein expression in the cells or tissues where the microorganism accumulates. Among the viruses provided are attenuated pox viruses that contain a modified TK and HA gene and a modified F3 gene or locus that corresponds to the F3 gene in vaccinia. In particular, provided are recombinant vaccina viruses that contain a modified TK and HA gene and optionally a modified F3 gene or locus, wherein the resulting virus does not accumulate to toxic levels in non-targeted organs. Vaccinia viruses where the TK gene and F3 gene are modified and vaccinia viruses where the HA and F3 gene are modified, and

viruses where all three genes are modified are provided. Modification includes inactivation by insertion, deletion or replacement of one or more nucleotide bases whereby an activity or product of the virus is altered. Included among the alterations is insertion of heterologous nucleic acid, such as therapeutic protein-encoding nucleic acids.

In exemplary embodiments, the vaccinia viruses are Lister strain viruses, particularly LIVP strain viruses (LIVP refers to the Lister virus from the Institute of Viral Preparations, Moscow, Russia, the original source for this now widely disseminated virus strain). Modifications include modification of the virus at the unique *Not*I site in the locus designed F3. In particular, the modification is at position 35 of the F3 gene or at position 1475 inside of the HindIII-F fragment of vaccinia virus DNA strain LIVP.

The heterologous nucleic acid can include regulatory sequence operatively linked to the nucleic acid encoding the protein. Regulatory sequences include promoters, such as the vaccinia virus early/late promoter p7.5 and an early/late vaccinia pE/L promoter. The heterologous nucleic acid in the microorganism can encode a detectable protein or a product capable of inducing a detectable signal. Inclusion of detectable protein or a product that can generate a detectable signal permits monitoring of the distribution of the administered microorganism as well as monitoring therapeutic efficacy, since the microorganism will be eliminated when the immunoprivileged cells are eliminated.

Host cells containing the recombinant viruses, such as the triple mutant vaccinia virus exemplified herein are provided. Also contemplated are tumor cells that contain any of the microorganisms provided herein or used in the methods.

Pharmaceutical composition containing the microorganisms in a pharmaceutically acceptable vehicle for use in the methods herein are provided. The pharmaceutical compositions can be formulated for any mode of administration, including, but not limited to systemic administration such as for intravenous administration or is formulated. The compositions can contain a delivery

vehicle, such as a lipid-based carrier, including liposomes and micelles associated with the microorganism.

Also provided are methods (and uses of the microorganisms) for eliminating immunoprivileged cells, such as tumor cells in an animal, by administering the pharmaceutical compositions to an animal, whereby the virus accumulates in the immunoprivileged cells, thereby mediating autoimmunization resulting in elimination of the cells or a reduction in their number.

Therapeutic methods for eliminating immunoprivileged cells or tissues, in an animal, by administering a microorganism to an animal, where the microorganism accumulates in the immunoprivileged cells; the microorganism does not accumulate in unaffected organs and tissues and has low toxicity in the animal; and the microorganism results in leakage of the cell membranes in the immunoprivileged cells, whereby the animal produces autoantibodies against the cells or products of the cells are provided. These methods include tumor treatment, treatment for inflammatory conditions, including wounds, and proliferative disorders, including psoriasis, cancers, diabetic retinapathies, restenosis and other such disorders. It is desirable for the microorganisms to not accumulate in unaffected organs, particularly the ovaries or testes.

The microorganisms attenuated bacteria, an attenuated viruses and mammalian cells, such as pox viruses and other cytoplasmic viruses, bacteria such as vibrio, E. coli, salmonella, streptococcus and listeria and mammalian cells, such as immune cells, including B cells and lymphocytes, such as T cells, and stem cells.

Methods for a recombinant vaccinia virus by : (a) generating (i) a vaccinia shuttle plasmid containing the modified F3 gene inserted at restriction site x and (ii) a dephosphorylated wt VV (VGL) DNA digested at a restriction site; transfecting host cells infected with psoralen -UV (PUV)-inactivated helper VV (VGL) with a mixture of constructs (i) and (ii) of step a; and (c) isolating the recombinant vaccinia viruses from the transfectants. Host cells include CV-1 cells.

Also provided are methods for production of a polypeptide or RNA or compound, such as a cellular product and uses of the microorganism therefore are provided. Such methods can include the steps of : (a) administering a microorganism containing nucleic acid encoding the polypeptide or RNA or producing the product compound to tumor-bearing animal, where the microorganism accumulates in the immunoprivileged cells; and the microorganism does not accumulate to toxic levels in organs and tissues that do not comprise immunoprivileged cells or tissues; (b) harvesting the tumor tissue from the animal; and (c) isolating the polypeptide or RNA or compound from the tumor.

As noted, the microorganisms include eukaryotic cells, prokaryotic cells and viruses,such as a cytoplasmic virus or an attenuated bacterium or a stem cell or an immune cell. The bacterium can be selected from among attenuated *vibrio, E. coli*, listeria, salmonella and streptococcus strains. The microorganism can express or produce detectable products, such as a fluorescent protein (*i.e.*, green, red and blue fluorescent proteins and modified variants thereof), and/or luciferase which, when contacted with a Lucifer produces light, and also can encode additional products, such as therapeutic products. In the methods and uses provided herein, the animals can be non-human animals or can include humans.

Also provided are methods for simultaneously producing a polypeptide. RNA molecule or cellular compound and an antibody that specifically reacts with the polypeptide, RNA molecule or compound, by: a) administering a microorganism to a tumor-bearing animal, wherein the microorganism expresses or produces the compound, polypeptide or RNA molecule; and b) isolating the antibody from serum in the animal. The method optionally includes, after step a) harvesting the tumor tissue from the animal; and isolating the polypeptide, RNA molecule or cellular compound from the tumor tissue.

Also provided are methods for eliminating immunoprivileged cells or tissues in an animal, such as tumor cells, and uses of the microorganisms therefore by administering at least two microorganisms, wherein the microorganisms are administered simultaneously, sequentially or intermittently, wherein the

microorganisms accumulate in the immunoprivileged cells, whereby the animal is autoimmunized against the immunoprivileged cells or tissues.

Uses of at least two microorganism for formulation of a medicament for elimination of immunoprivileged cells or tissues, wherein they accumulate in the immunoprivileged cells, whereby the animal is autoimmunized against the immunoprivileged cells or tissues are provided. Combinations containing at least two microorganisms formulated for administration to an animal for elimination of immunoprivileged cells or tissues are provided. Kits containing packaged combination optionally with instructions for administration and other reagents are provided.

Uses of a microorganism encoding heterologous nucleic acid for inducing autoimmunization against products produced in immunoprivileged cells, wherein, when administered, the microorganism accumulates in immunoprivileged tissues and does not accumulate or accumulates at a sufficiently low level in other tissues or organs to be non-toxic to an animal containing the immunoprivileged tissues are provided.

Methods for the production of antibodies against products produced in immunoprivileged tissues or cells by: (a) administering a microorganism containing nucleic acid encoding a selected protein or RNA into an animal containing the immunoprivileged tissues or cells ; and (b) isolating antibodies against the protein or RNA from the blood or serum of the animal are provided.

Also provided are methods for inhibiting growth of immunoprivileged cells or tissue in a subject by: (a) administering to a subject a modified microorganism, wherein the modified microorganism encodes a detectable gene product; (b) monitoring the presence of the detectable gene product in the subject until the detectable gene product is substantially present only in immunoprivileged tissue or cells of a subject; and (c) administering to a subject a therapeutic compound that works in conjunction with the microorganism to inhibit growth of immunoprivileged cells or tissue or by: (a) administering to a subject a modified microorganism that encodes a detectable gene product; (b) administering to a subject

a therapeutic substance that reduces the pathogenicity of the microorganism; (c) monitoring the presence of the detectable gene product in the subject until the detectable gene product is substantially present only in immunoprivileged tissue or cells of a subject; and (d) terminating or suspending administration of the therapeutic compound, whereby the microorganism increases in pathogenicity and the growth of the immunoprivileged cells or tissue is inhibited.

DESCRIPTION OF THE FIGURES

Figure 1A: Schematic representation of the recombinant vaccinia virus RVGL8 used. The recombinant vaccinia virus RVGL8 was constructed by using the in vivo recombination method described in Example 1. The complex of wild-type vaccinia virus DNA digested with *NotI* and non-digested plasmid DNA pNZ2 was transfected for in vivo recombination into PUV-VV-infected cells. VGL, wild type vaccinia virus (strain Lister ATCC VR-1549); RVGL8, recombinant vaccinia virus encoding the lacZ gene in the *NotI* site; Not_L and Not_R, left and right segments of unique NotI restriction site in vaccinia virus genome.; pE/L, synthetic early/late vaccinia virus promoter; p7.5, early/late vaccinia virus promoter; lacZ, lacZ gene of *E. coli*.

Figure 1B: Schematic of the various vaccinia strains described in the Examples. Results achieved the viruses are described in the Examples.

Figure 2 sets forth a flow chart for a method for producing products, such as nucleic acid molecules, proteins and metabolic compounds or other cellular products in tumors.

DETAILED DESCRIPTION

A. Definitions

B. Microorganisms for Tumor-Specific Therapy

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1. Characteristics

a. Attenuated

i. Reduced toxicity

ii. Accumulate in tumor, not substantially in other organs

iii. Ability to Elicit or Enhance Immune Response to Tumor Cell

iv. Balance of Pathogenicity and Release of Tumor Antigens

b. Immunogenicity
c. Replication Competent
d. Genetic Variants
i. Modfied Characteristics
ii. Exogenous Gene Expression
iii. Detectable gene product
iv. Therapeutic gene product
v. Expressing a superantigen
vi. Expressing a gene product to be harvested
2. Viruses
a. Cytoplasmic viruses
i. Poxviruses
a. Vaccinia Virus
b. Modified Vaccinia Viruses
c. The F3 Gene
d. Multiple Modifications
e. The Lister Strain
ii. Other cytoplasmic viruses
b. Adenovirus, Herpes, Retroviruses
3. Bacteria
a. Aerobic bacteria
b. Anaerobic bacteria
4. Eukaryotic cells
C. Methods for Making an Attenuated Microorganism
1. Genetic Modifications
2. Screening for above characteristics
3. Methods for developing such a microorganism in humans
D. Therapeutic Methods
1. Administration
a. Steps prior to administering the microorganism
b. Mode of administration
c. Dosage
d. Number of administrations
e. Co-administrations
i. Administering a plurality of microorganisms
ii. Therapeutic compounds
f. State of subject
2. Monitoring
a. Monitoring microorganismal gene expression
b. Monitoring tumor size
c. Monitoring antibody titer
d. Monitoring general health diagnostics
e. Monitoring coordinated with treatment
E. Methods of Producing Gene Products and Antibodies
1. Production of Recombinant Proteins and RNA molecules
2. Production of Antibodies
F. Pharmaceutical Compositions, combinations and kits
1. Pharmaceutical Compositions
2. Host Cells
3. Combinations
4. Kits
G. Examples

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information is known and can be readily accessed, such as by searching the internet and/or appropriate databases. Reference thereto evidences the availability and public dissemination of such information.

As used herein, microorganisms refers to isolated cells or viruses, including eukaryotic cells, such as mammalian cells, viruses and bacteria. The microorganisms are modified or selected for their ability to accumulate in tumors and other immunoprivileged cells and tissues, and to minimize accumulation in other tissues or organs. Accumulation occurs by virtue of selection or modification of the microorganisms for particular traits or by proper selection of cells. The microorganism can be further modified to alter a trait thereof and/or to deliver a gene product. The microorganisms provided herein are typically modified relative to wild type to exhibit one or more characteristics such as reduced pathogenicity, reduced toxicity, preferential accumulation in tumor relative to normal organs or tissues, increased immunogenicity, increased ability to elicit or enhance an immune response to tumor cells, increased lytic or tumor cell killing capacity, decreased lytic or tumor cell killing capacity.

As used herein, immunoprivileged cells and tissues refer to cells and tissues, such as solid tumors and wounded tissues, which are sequestered from the immune system. Generally administration of a microorganism elicits an immune response that clears the microorganism; immunoprivileged sites, however, are shielded or sequestered from the immune response, permitting the microorganisms to survive

and generally to replicate. Immunoprivileged tissues include inflamed tissues, such as wounded tissues, and proliferating tissues, such as tumor tissues.

As used herein, "modified" with reference to a gene refers to a deleted gene, or a gene encoding a gene product having one or more truncations, mutations, insertions or deletions, typically accompanied by at least a change, generally a partial loss of function.

As used herein F3 gene refers to a gene or locus in a virus, such as a vaccinia virus, that corresponds to the F3 gene of vaccinia virus strain LIVP. This includes the F3 gene of any vaccinia virus strain or poxvirus encoding a gene product having substantially the same or at least a related biological function or locus in the genome. F3 genes encompassed herein typically have at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity along the full length of the sequence of nucleotides set forth in SEQ ID No:1. The proteins encoded by F3 genes encompassed herein typically have at least about 50%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 85%, at least about 90%, at least about 93%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity to the sequence of amino acids set forth SEQ ID No. 2 along the full length thereof. Also included are corresponding loci in other viruses that when modified or eliminated result in reduced toxicity and/or enhanced accumulation in tumors (compared to non-tumorous cells, tissues and organs). The corresponding loci in other viruses equivalent to the F3 gene in LIVP can be determined by the structural location of the gene in the viral genome: the LIVP F3 gene is located on the HindIII-F fragment of vaccinia virus between open reading frames F14L and F15L as defined by Goebel *et al.*, Virology (1990) 179:247-266, and in the opposite orientation of ORFs F14L and F15L; thus corresponding loci in other viruses such as poxviruses including orthopoxviruses are included.

As used herein, attenuate toxicity of a microorganism means to reduce or eliminate deleterious or toxic effects to a host upon administration of the microorganism compared to the unattenuated microorganism.

As use herein, a microorganism with low toxicity means that upon administration a microorganism does not accumulate in organs and tissues in the host to an extent that results in damage or harm to organs or that impact on survival of the host to a greater extent than the disease being treated does.

As used herein, subject (or organism) refers to an animal, including a human being.

As used herein, animal includes any animal, such as, but are not limited to primates including humans, gorillas and monkeys; rodents, such as mice and rats; fowl, such as chickens; ruminants, such as goats, cows, deer, sheep; ovine, and other animals including pigs, horses, cats, dogs, and rabbits. Non-human animals exclude humans as the contemplated animal.

As used herein, accumulation of a microorganism in a targeted tissue refers to the distribution of the microorganism throughout the organism after a time period long enough for the microbes to infect the host's organs or tissues. As one skilled in the art will recognize, the time period for infection of a microbe will vary depending on the microbe, the targeted organ(s) or tissue(s), the immunocompetence of the host, and dosage. Generally, accumulation can be determined at time point from about 1 day to about 1 week after infection with the microbes. For purposes herein, the microorganisms preferentially accumulate in the target tissue, such as a tumor, but are cleared from other tissues and organs in the host to the extent that toxicity of the microorganism is mild or tolerable and at most not fatal.

As used herein, preferential accumulation refers to accumulation of a microorganism at a first location at a higher level than accumulation at a second location. Thus, a microorganism that preferentially accumulates in immunoprivileged tissue such as tumor relative to normal tissues or organs refers to a microorganism that accumulates in immunoprivileged tissue such as tumor at a higher level than the microorganism accumulates in normal tissues or organs.

As used herein, a "compound" produced in a tumor or other immunoprivileged site refers to any compound that is produced in the tumor by virtue of the presence of an introduced microorganism, generally a recombinant microorganism, expressing one or more genes. For example, a compound produced in a tumor can be, for example, a metabolite, an encoded polypeptide or RNA, or compound that is generated by a recombinant polypeptide (*e.g.*, enzyme) and the cellular machinery of the tumor or immunoprivileged tissue or cells.

As used herein, a delivery vehicle for administration refers to a lipid-based or other polymer based composition, such as liposome, micelle or reverse micelle, that associates with an agent, such as a microorganism provided herein, for delivery into a host animal.

As used herein, the term "viral vector" is used according to its art-recognized meaning. It refers to a nucleic acid vector construct that includes at least one element of viral origin and can be packaged into a viral vector particle. The viral vector particles can be used for the purpose of transferring DNA, RNA or other nucleic acids into cells either in vitro or in vivo. Viral vectors include, but are not limited to, retroviral vectors, vaccinia vectors, lentiviral vectors, herpes virus vectors (*e.g.*, HSV), baculoviral vectors, cytomegalovirus (CMV) vectors, papillomavirus vectors, simian virus (SV40) vectors, semliki forest virus vectors, phage vectors, adenoviral vectors, and adeno-associated viral (AAV) vectors.

As used herein, oncolytic viruses refer to viruses that replicate selectively in tumor cells.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from, *e.g.*, infection or genetic defect, and characterized by identifiable symptoms.

As used herein, neoplasm (neoplasia) refers to abnormal new growth, and thus means the same as tumor, which can be benign or malignant. Unlike hyperplasia, neoplastic proliferation persists even in the absence of the original stimulus.

As used herein, neoplastic disease refers to any disorder involving cancer, including tumor development, growth, metastasis and progression.

As used herein, cancer is a general term for diseases caused by or characterized by any type of malignant tumor.

As used herein, malignant, as applies to tumors, refers to primary tumors that have the capacity of metastasis with loss of growth control and positional control.

As used herein, metastasis refers to a growth of abnormal or neoplastic cells distant from the site primarily involved by the morbid process.

As used herein, an anti-cancer agent or compound (used interchangeably with "anti-tumor or anti-neoplastic agent") refers to any agents or compounds used in anti-cancer treatment. These include any agents, when used alone or in combination with other compounds, that can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with neoplastic disease, tumors and cancer, and can be used in methods, combinations and compositions provided herein. Exemplary anti-neoplastic agents include the microorganism provided herein used singly or in combination and/or in combination with other agents, such as alkylating agents, antimetabolite, certain natural products, platinum coordination complexes, anthracenediones, substituted ureas, methylhydrazine derivatives, adrenocortical suppressants, certain hormones, antagonists and anti-cancer polysaccharides.

In general, for practice of the methods herein and when using the microorganisms provided herein, the original tumor is not excised, but is employed to accumulate the administered microorganism and as the cells become leaky or lyse to become an antigen or other product factor. The antigens can serve to elicit an immune response in the host. The antigens and products can be isolated from the tumor.

As used herein, angiogenesis is intended to encompass the totality of processes directly or indirectly involved in the establishment and maintenance of new vasculature (neovascularization), including, but not limited to,

neovascularization associated with tumors and neovascularization associated with wounds.

As used herein, by homologous means about greater than 25% nucleic acid sequence identity, such as 25%, 40%, 60%, 70%, 80%, 90% or 95%. If necessary the percentage homology will be specified. The terms "homology" and "identity" are often used interchangeably but homology for proteins can include conservative amino acid changes. In general, sequences (protein or nucleic acid) are aligned so that the highest order match is obtained (see, *e.g.*: Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; Carillo *et al.* (1988) SIAM J Applied Math 48:1073). By sequence identity, the number of identical amino acids is determined by standard alignment algorithm programs, and used with default gap penalties established by each supplier. Substantially homologous nucleic acid molecules would hybridize typically at moderate stringency or at high stringency all along the length of the nucleic acid or along at least about 70%, 80% or 90% of the full length nucleic acid molecule of interest. Also provided are nucleic acid molecules that contain degenerate codons in place of codons in the hybridizing nucleic acid molecule. (For proteins, for determination of homology conservative amino acids can be aligned as well as identical amino acids; in this case percentage of identity and percentage homology vary). Whether any two nucleic acid molecules have nucleotide sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FASTA" program, using for example, the default parameters as in Pearson *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:2444 (other programs include the GCG program package (Devereux, J., *et al.*, Nucleic Acids Research 12(I):387 (1984)), BLASTP, BLASTN, FASTA Atschul, S.F., *et al.*, J Molec Biol

215:403 (1990); Guide to Huge Computers, Mrtin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo *et al.* (1988) SIAM J Applied Math 48:1073). For example, the BLAST function of the National Center for Biotechnology Information database can be used to determine identity. Other commercially or publicly available programs include, DNAStar "MegAlign" program (Madison, WI) and the University of Wisconsin Genetics Computer Group (UWG) "Gap" program (Madison WI)). Percent homology or identity of proteins and/or nucleic acid molecules can be determined, for example, by comparing sequence information using a GAP computer program (*e.g.*, Needleman *et al.* (1970) J. Mol. Biol. 48:443, as revised by Smith and Waterman ((1981) Adv. Appl. Math. 2:482).

Briefly, a GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986) Nucl. Acids Res. 14:6745, as described by Schwartz and Dayhoff, eds., ATLAS OF PROTEIN SEQUENCE AND STRUCTURE, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide.

As used herein, recitation that amino acids of a polypeptide correspond to amino acids in a disclosed sequence, such as amino acids set forth in the Sequence listing, refers to amino acids identified upon alignment of the polypeptide with the disclosed sequence to maximize identity or homology (where conserved amino acids are aligned) using a standard alignment algorithm, such as the GAP algorithm.

As used herein, the term "at least 90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared, no

more than 10% (*i.e.*, 10 out of 100) of amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, *e.g.*, 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. At the level of homologies or identities above about 85-90%, the result should be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

As used herein, primer refers to an oligonucleotide containing two or more deoxyribonucleotides or ribonucleotides, typically more than three, from which synthesis of a primer extension product can be initiated. Experimental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization and extension, such as DNA polymerase, and a suitable buffer, temperature and pH.

As used herein, chemiluminescence refers to a chemical reaction in which energy is specifically channeled to a molecule causing it to become electronically excited and subsequently to release a photon thereby emitting visible light. Temperature does not contribute to this channeled energy. Thus, chemiluminescence involves the direct conversion of chemical energy to light energy.

As used herein, luminescence refers to the detectable EM radiation, generally, UV, IR or visible EM radiation that is produced when the excited product of an exergic chemical process reverts to its ground state with the emission of light. Chemiluminescence is luminescence that results from a chemical reaction. Bioluminescence is chemiluminescence that results from a chemical reaction using biological molecules (or synthetic versions or analogs thereof) as substrates and/or enzymes.

As used herein, bioluminescence, which is a type of chemiluminescence, refers to the emission of light by biological molecules, particularly proteins. The essential condition for bioluminescence is molecular oxygen, either bound or free in the presence of an oxygenase, a luciferase, which acts on a substrate, a luciferin. Bioluminescence is generated by an enzyme or other protein (luciferase) that is an oxygenase that acts on a substrate luciferin (a bioluminescence substrate) in the presence of molecular oxygen, and transforms the substrate to an excited state, which, upon return to a lower energy level releases the energy in the form of light.

As used herein, the substrates and enzymes for producing bioluminescence are generically referred to as luciferin and luciferase, respectively. When reference is made to a particular species thereof, for clarity, each generic term is used with the name of the organism from which it derives, for example, bacterial luciferin or firefly luciferase.

As used herein, luciferase refers to oxygenases that catalyze a light emitting reaction. For instance, bacterial luciferases catalyze the oxidation of flavin mononucleotide (FMN) and aliphatic aldehydes, which reaction produces light. Another class of luciferases, found among marine arthropods, catalyzes the oxidation of Cypridina (Vargula) luciferin, and another class of luciferases catalyzes the oxidation of Coleoptera luciferin.

Thus, luciferase refers to an enzyme or photoprotein that catalyzes a bioluminescent reaction (a reaction that produces bioluminescence). The luciferases, such as firefly and Gaussia and Renilla luciferases, are enzymes which act catalytically and are unchanged during the bioluminescence generating reaction. The luciferase photoproteins, such as the aequorin photoprotein to which luciferin is non-covalently bound, are changed, such as by release of the luciferin, during bioluminescence generating reaction. The luciferase is a protein that occurs naturally in an organism or a variant or mutant thereof, such as a variant produced by mutagenesis that has one or more properties, such as thermal stability, that differ from the naturally-occurring protein. Luciferases and modified mutant or variant

forms thereof are well known. For purposes herein, reference to luciferase refers to either the photoproteins or luciferases.

Thus, reference, for example, to "Renilla luciferase" means an enzyme isolated from member of the genus Renilla or an equivalent molecule obtained from any other source, such as from another related copepod, or that has been prepared synthetically. It is intended to encompass Renilla luciferases with conservative amino acid substitutions that do not substantially alter activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and can be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al.* Molecular Biology of the Gene, 4th Edition, 1987, The Benjamin/Cummings Pub. co., p.224).

As used herein, "Aequora GFP" refers to GFPs from the genus Aequora and to mutants or variants thereof. Such variants and GFPs from other species are well known and are available and known to those of skill in the art. This nomenclature encompass GFPs with conservative amino acid substitutions that do not substantially alter activity and physical properties, such as the emission spectra and ability to shift the spectral output of bioluminescence generating systems. The luciferases and luciferin and activators thereof are referred to as bioluminescence generating reagents or components. Typically, a subset of these reagents will be provided or combined with an article of manufacture. Bioluminescence will be produced upon contacting the combination with the remaining reagents. Thus, as used herein, the component luciferases, luciferins, and other factors, such as O_2, Mg^{2+}, Ca^{2+} are also referred to as bioluminescence generating reagents (or agents or components).

As used herein, bioluminescence substrate refers to the compound that is oxidized in the presence of a luciferase, and any necessary activators, and generates light. These substrates are referred to as luciferins herein, are substrates that undergo oxidation in a bioluminescence reaction. These bioluminescence substrates include any luciferin or analog thereof or any synthetic compound with which a

luciferase interacts to generate light. Typical substrates include those that are oxidized in the presence of a luciferase or protein in a light-generating reaction. Bioluminescence substrates, thus, include those compounds that those of skill in the art recognize as luciferins. Luciferins, for example, include firefly luciferin, Cypridina (also known as Vargula) luciferin (coelenterazine), bacterial luciferin, as well as synthetic analogs of these substrates or other compounds that are oxidized in the presence of a luciferase in a reaction the produces bioluminescence.

As used herein, capable of conversion into a bioluminescence substrate means susceptible to chemical reaction, such as oxidation or reduction, that yields a bioluminescence substrate. For example, the luminescence producing reaction of bioluminescent bacteria involves the reduction of a flavin mononucleotide group (FMN) to reduced flavin mononucleotide (FMNH2) by a flavin reductase enzyme. The reduced flavin mononucleotide (substrate) then reacts with oxygen (an activator) and bacterial luciferase to form an intermediate peroxy flavin that undergoes further reaction, in the presence of a long-chain aldehyde, to generate light. With respect to this reaction, the reduced flavin and the long chain aldehyde are substrates.

As used herein, a bioluminescence generating system refers to the set of reagents required to conduct a bioluminescent reaction. Thus, the specific luciferase, luciferin and other substrates, solvents and other reagents that can be required to complete a bioluminescent reaction form a bioluminescence system. Thus a bioluminescence generating system refers to any set of reagents that, under appropriate reaction conditions, yield bioluminescence. Appropriate reaction conditions refers to the conditions necessary for a bioluminescence reaction to occur, such as pH, salt concentrations and temperature. In general, bioluminescence systems include a bioluminescence substrate, luciferin, a luciferase, which includes enzymes luciferases and photoproteins, and one or more activators. A specific bioluminescence system may be identified by reference to the specific organism from which the luciferase derives; for example, the Renilla bioluminescence system includes a Renilla luciferase, such as a luciferase isolated from the Renilla or

produced using recombinant means or modifications of these luciferases. This system also includes the particular activators necessary to complete the bioluminescence reaction, such as oxygen and a substrate with which the luciferase reacts in the presence of the oxygen to produce light.

As used herein, a fluorescent protein refers to a protein that possesses the ability to fluoresce (*i.e.*, to absorb energy at one wavelength and emit it at another wavelength). For example, a green fluorescent protein refers to a polypeptide that has a peak in the emission spectrum at about 510 nm.

As used herein, genetic therapy or gene therapy involves the transfer of heterologous nucleic acid, such as DNA, into certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The nucleic acid, such as DNA, is introduced into the selected target cells, such as directly or in a vector or other delivery vehicle, in a manner such that the heterologous nucleic acid, such as DNA, is expressed and a therapeutic product encoded thereby is produced. Alternatively, the heterologous nucleic acid, such as DNA, can in some manner mediate expression of DNA that encodes the therapeutic product, or it can encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy also can be used to deliver nucleic acid encoding a gene product that replaces a defective gene or supplements a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid can encode a therapeutic compound, such as a growth factor inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous nucleic acid, such as DNA, encoding the therapeutic product can be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof. Genetic therapy also can involve delivery of an inhibitor or repressor or other modulator of gene expression.

As used herein, heterologous nucleic acid is nucleic acid that is not normally produced *in vivo* by the microorganism from which it is expressed or that is produced by a microorganism but is at a different locus or expressed differently or that mediates or encodes mediators that alter expression of endogenous nucleic acid, such as DNA, by affecting transcription, translation, or other regulatable biochemical processes. Heterologous nucleic acid is often not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically. Heterologous nucleic acid, however, can be endogenous, but is nucleic acid that is expressed from a different locus or altered in its expression or sequence. Generally, although not necessarily, such nucleic acid encodes RNA and proteins that are not normally produced by the cell or in the same way in the cell in which it is expressed. Heterologous nucleic acid, such as DNA, also can be referred to as foreign nucleic acid, such as DNA. Thus, heterologous nucleic acid or foreign nucleic acid includes a nucleic acid molecule not present in the exact orientation or position as the counterpart nucleic acid molecule, such as DNA, is found in a genome. It also can refer to a nucleic acid molecule from another organism or species (*i.e.*, exogenous). Any nucleic acid, such as DNA, that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which the nucleic acid is expressed is herein encompassed by heterologous nucleic acid; heterologous nucleic acid includes exogenously added nucleic acid that also is expressed endogenously. Examples of heterologous nucleic acid include, but are not limited to, nucleic acid that encodes traceable marker proteins, such as a protein that confers drug resistance, nucleic acid that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and nucleic acid, such as DNA, that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous nucleic acid can be secreted or expressed on the surface of the cell in which the heterologous nucleic acid has been introduced.

As used herein, a therapeutically effective product for gene therapy is a product that is encoded by heterologous nucleic acid, typically DNA, (or an RNA product such as dsRNA, RNAi, including siRNA, that, upon introduction of the

nucleic acid into a host, a product is expressed that ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures the disease. Also included are biologically active nucleic acid molecules, such as RNAi and antisense.

As used herein, cancer or tumor treatment or agent refers to any therapeutic regimen and/or compound that, when used alone or in combination with other treatments or compounds, can alleviate, reduce, ameliorate, prevent, or place or maintain in a state of remission of clinical symptoms or diagnostic markers associated with deficient angiogenesis.

As used herein, nucleic acids include DNA, RNA and analogs thereof, including peptide nucleic acids (PNA) and mixtures thereof. Nucleic acids can be single or double-stranded. When referring to probes or primers, which are optionally labeled, such as with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are provided. Such molecules are typically of a length such that their target is statistically unique or of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleic acids long.

As used herein, operative linkage of heterologous nucleic to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences refers to the relationship between such nucleic acid, such as DNA, and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. Thus, operatively linked or operationally associated refers to the functional relationship of nucleic acid, such as DNA, with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences.

For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it can be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that can interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak J. Biol. Chem. 266:19867-19870 (1991)) can be inserted immediately 5' of the start codon and can enhance expression. The desirability of (or need for) such modification can be empirically determined.

As used herein, a sequence complementary to at least a portion of an RNA, with reference to antisense oligonucleotides, means a sequence of nucleotides having sufficient complementarity to be able to hybridize with the RNA, generally under moderate or high stringency conditions, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA (or dsRNA) can thus be tested, or triplex formation can be assayed. The ability to hybridize depends on the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an encoding RNA it can contain and still form a stable duplex (or triplex, as the case can be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

As used herein, amelioration of the symptoms of a particular disorder such as by administration of a particular pharmaceutical composition, refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein, antisense polynucleotides refer to synthetic sequences of nucleotide bases complementary to mRNA or the sense strand of double-stranded

DNA. Admixture of sense and antisense polynucleotides under appropriate conditions leads to the binding of the two molecules, or hybridization. When these polynucleotides bind to (hybridize with) mRNA, inhibition of protein synthesis (translation) occurs. When these polynucleotides bind to double-stranded DNA, inhibition of RNA synthesis (transcription) occurs. The resulting inhibition of translation and/or transcription leads to an inhibition of the synthesis of the protein encoded by the sense strand. Antisense nucleic acid molecules typically contain a sufficient number of nucleotides to specifically bind to a target nucleic acid, generally at least 5 contiguous nucleotides, often at least 14 or 16 or 30 contiguous nucleotides or modified nucleotides complementary to the coding portion of a nucleic acid molecule that encodes a gene of interest.

As used herein, antibody refers to an immunoglobulin, whether natural or partially or wholly synthetically produced, including any derivative thereof that retains the specific binding ability of the antibody. Hence antibody includes any protein having a binding domain that is homologous or substantially homologous to an immunoglobulin binding domain. Antibodies include members of any immunoglobulin class, including IgG, IgM, IgA, IgD and IgE.

As used herein, antibody fragment refers to any derivative of an antibody that is less then full length, retaining at least a portion of the full-length antibody's specific binding ability. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab)2, single-chain Fvs (scFV), FV, dsFV diabody and Fd fragments. The fragment can include multiple chains linked together, such as by disulfide bridges. An antibody fragment generally contains at least about 50 amino acids and typically at least 200 amino acids.

As used herein, a Fv antibody fragment is composed of one variable heavy chain domain (VH) and one variable light chain domain linked by noncovalent interactions.

As used herein, a dsFV refers to an Fv with an engineered intermolecular disulfide bond, which stabilizes the VH-VL pair.

As used herein, a F(ab)2 fragment is an antibody fragment that results from digestion of an immunoglobulin with pepsin at pH 4.0-4.5; it can be recombinantly produced to produce the equivalent fragment.

As used herein, Fab fragments are antibody fragments that result from digestion of an immunoglobulin with papain; it can be recombinantly produced to produce the equivalent fragment.

As used herein, scFVs refer to antibody fragments that contain a variable light chain (VL) and variable heavy chain (VH) covalently connected by a polypeptide linker in any order. The linker is of a length such that the two variable domains are bridged without substantial interference. Included linkers are (Gly-Ser)n residues with some Glu or Lys residues dispersed throughout to increase solubility.

As used herein, humanized antibodies refer to antibodies that are modified to include human sequences of amino acids so that administration to a human does not provoke an immune response. Methods for preparation of such antibodies are known. For example, to produce such antibodies, the encoding nucleic acid in the hybridoma or other prokaryotic or eukaryotic cell, such as an E. coli or a CHO cell, that expresses the monoclonal antibody is altered by recombinant nucleic acid techniques to express an antibody in which the amino acid composition of the non-variable region is based on human antibodies. Computer programs have been designed to identify such non-variable regions.

As used herein, diabodies are dimeric scFV; diabodies typically have shorter peptide linkers than scFvs, and they generally dimerize.

As used herein, production by recombinant means by using recombinant DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein the term assessing or determining is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the activity of a product, and also of obtaining an index, ratio, percentage, visual

or other value indicative of the level of the activity. Assessment can be direct or indirect.

As used herein, biological activity refers to the *in vivo* activities of a compound or microorganisms or physiological responses that result upon *in vivo* administration thereof or of composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities can be observed in *in vitro* systems designed to test or use such activities.

As used herein, an effective amount of a microorganism or compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such an amount can be administered as a single dosage or can be administered according to a regimen, whereby it is effective. The amount can cure the disease but, typically, is administered in order to ameliorate the symptoms of the disease. Repeated administration can be required to achieve the desired amelioration of symptoms.

As used herein equivalent, when referring to two sequences of nucleic acids, means that the two sequences in question encode the same sequence of amino acids or equivalent proteins. When equivalent is used in referring to two proteins or peptides or other molecules , it means that the two proteins or peptides have substantially the same amino acid sequence with only amino acid substitutions (such as,but not limited to, conservative changes) or structure and the changes do not substantially alter the activity or function of the protein or peptide. When equivalent refers to a property, the property does not need to be present to the same extent (*e.g.*, two peptides can exhibit different rates of the same type of enzymatic activity), but the activities are usually substantially the same. Complementary, when referring to two nucleotide sequences, means that the two sequences of nucleotides are capable of hybridizing, typically with less than 25%, 15% or 5% mismatches between opposed nucleotides. If necessary, the percentage of complementarity will be specified. Typically the two molecules are selected such that they will hybridize under conditions of high stringency.

As used herein, an agent or compound that modulates the activity of a protein or expression of a gene or nucleic acid either decreases or increases or otherwise alters the activity of the protein or, in some manner, up- or down-regulates or otherwise alters expression of the nucleic acid in a cell.

As used herein, a method for treating or preventing neoplastic disease means that any of the symptoms, such as the tumor, metastasis thereof, the vascularization of the tumors or other parameters by which the disease is characterized are reduced, ameliorated, prevented, placed in a state of remission, or maintained in a state of remission. It also means that the hallmarks of neoplastic disease and metastasis can be eliminated, reduced or prevented by the treatment. Non-limiting examples of the hallmarks include uncontrolled degradation of the basement membrane and proximal extracellular matrix, migration, division, and organization of the endothelial cells into new functioning capillaries, and the persistence of such functioning capillaries.

As used herein, a prodrug is a compound that, upon in vivo administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. To produce a prodrug, the pharmaceutically active compound is modified such that the active compound is regenerated by metabolic processes. The prodrug can be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter other characteristics or properties of a drug. By virtue of knowledge of pharmacodynamic processes and drug metabolism in vivo, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, *e.g.*, Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, New York, pages 388-392).

As used herein, a promoter region or promoter element or regulatory region refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In

addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences can be cis acting or can be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, can be constitutive or regulated. Exemplary promoters contemplated for use in prokaryotes include the bacteriophage T7 and T3 promoters.

As used herein, a receptor refers to a molecule that has an affinity for a ligand. Receptors can be naturally-occurring or synthetic molecules. Receptors also can be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors can be used in their unaltered state or bound to other polypeptides, including as homodimers. Receptors can be attached to, covalently or noncovalently, or in physical contact with, a binding member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell membrane receptors surface receptors and internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

As used herein, sample refers to anything that can contain an analyte for which an analyte assay is desired. The sample can be a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological tissues are aggregates of cells, usually of a particular kind together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s).

As used herein: stringency of hybridization in determining percentage mismatch is as follows:

1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C

2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C

3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C

Those of skill in this art know that the washing step selects for stable hybrids and also know the ingredients of SSPE (see, *e.g.*, Sambrook, E.F. Fritsch, T. Maniatis, in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), vol. 3, p. B.13, see, also, numerous catalogs that describe commonly used laboratory solutions). SSPE is pH 7.4 phosphate-buffered 0.18 M NaCl. Further, those of skill in the art recognize that the stability of hybrids is determined by Tm, which is a function of the sodium ion concentration and temperature : (Tm = 81.5o C-16.6(log10[Na+]) + 0.41(%G+C)-600/l)), so that the only parameters in the wash conditions critical to hybrid stability are sodium ion concentration in the SSPE (or SSC) and temperature. Any nucleic acid molecules provided herein can also include those that hybridize under conditions of at least low stringency, generally moderate or high stringency, along at least 70, 80, 90% of the full length of the disclosed molecule. It is understood that equivalent stringencies can be achieved using alternative buffers, salts and temperatures. By way of example and not limitation, procedures using conditions of low stringency are as follows (see also Shilo and Weinberg, Proc. Natl. Acad. Sci. USA 78:6789-6792 (1981)):

Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500µg/ml denatured salmon sperm DNA (10X SSC is 1.5 M sodium chloride, and 0.15 M sodium citrate, adjusted to a pH of 7). Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Filters are blotted dry and

exposed for autoradiography. If necessary, filters are washed for a third time at 65-68øC and reexposed to film. Other conditions of low stringency which can be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

By way of example and not way of limitation, procedures using conditions of moderate stringency include, for example, but are not limited to, procedures using such conditions of moderate stringency are as follows: Filters containing DNA are pretreated for 6 hours at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18- 20 hours at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency which can be used are well-known in the art. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.1% SDS. By way of example and not way of limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100µg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 minutes before autoradiography. Other conditions of high stringency which can be used are well known in the art.

The term substantially identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 60% or 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% , 96%, 97%, 98%, 99% or greater identity.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound can, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, a molecule, such as an antibody, that specifically binds to a polypeptide typically has a binding affinity (Ka) of at least about 10^6 l/mol, 10^7 l/mol, 10^8 l/mol, 10^9 l/mol, 10^{10} l/mol or greater and binds to a protein of interest generally with at least 2-fold, 5-fold, generally 10-fold or even 100-fold or greater, affinity than to other proteins. For example, an antibody that specifically binds to the protease domain compared to the full-length molecule, such as the zymogen form, binds with at least about 2-fold, typically 5-fold or 10-fold higher affinity, to a polypeptide that contains only the protease domain than to the zymogen form of the full-length. Such specific binding also is referred to as selective binding. Thus, specific or selective binding refers to greater binding affinity (generally at least 2-fold, 5-fold, 10-fold or more) to a targeted site or locus compared to a non-targeted site or locus.

As used herein, the terms a therapeutic agent, therapeutic compound, therapeutic regimen, or chemotherapeutic include conventional drugs and drug therapies, including vaccines, which are known to those skilled in the art.

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the microorganisms described and provided herein.

As used herein, proliferative disorders include any disorders involving abnormal proliferation of cells. Such disorders include, but are not limited to, neoplastic diseases, psoriasis, restenosis, macular degeneration, diabetic retinopathies, inflammatory responses and disorders, including wound healing responses.

As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous nucleic acid into cells for either expression or replication thereof. The vectors typically remain episomal, but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vectors are well known to those of skill in the art. An expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, a combination refers to any association between two or among more items.

As used herein, a composition refers to any mixture. It can be a solution, a suspension, an emulsion, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

As used herein, a kit is a packaged combination optionally including instructions for use of the combination and/or other reactions and components for such use.

For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow.

B. Microorganisms for Tumor-Specific Therapy

Provided herein are microorganisms, and methods for making and using such microorganisms for therapy of neoplastic disease and other proliferative disorders and inflammatory disorders. The microbe (or microorganism)-mediated treatment methods provided herein involve administration of microorganisms to hosts, accumulation of the microorganism in the targeted cell or tissue, such as in a tumor, resulting in leaking or lysing of the cells, whereby an immune response against leaked or released antigens is mounted, thereby resulting in an inhibition of the tissues or cells in which the microorganism accumulates.

In addition to the gene therapeutic methods of cancer treatment, live attenuated microorganisms can be used for vaccination, such as in cancer vaccination or antitumor immunity. Immunization, for example, against a tumor can include a tumor-specific T-cell-mediated response through microbe-delivered antigens or cytokines. To do so, the microbes can be specifically targeted to the tumor tissues, with minimal infection to any other key organs and also can be modified or provided to produce the antigens and/or cytokines.

The microorganisms provided herein and the use of such microorganisms herein can accumulate in immunoprivileged cells or immunoprivileged tissues, including tumors and/or metastases, and also including wounded tissues and cells. While the microorganisms provided herein can typically be cleared from the subject to whom the microorganisms are administered by activity of the subject's immune system, microorganisms can nevertheless accumulate, survive and proliferate in

immunoprivileged cells and tissues such as tumors because such immunoprivileged areas are sequestered from the host's immune system. Accordingly, the methods provided herein, as applied to tumors and/or metastases, and therapeutic methods relating thereto, can readily be applied to other immunoprivileged cells and tissues, including wounded cells and tissues.

1. Characteristics

The microorganisms provided herein and used in the methods herein are attenuated, immunogenic, and replication competent.

a. Attenuated

The microbes used in the methods provided herein are typically attenuated. Attenuated microbes have a decreased capacity to cause disease in a host. The decreased capacity can result from any of a variety of different modifications to the ability of a microbe to be pathogenic. For example, a microbe can have reduced toxicity, reduced ability to accumulate in non-tumorous organs or tissue, reduced ability to cause cell lysis or cell death, or reduced ability to replicate compared to the non-attenuated form thereof. The attenuated microbes provided herein, however, retain at least some capacity to replicate and to cause immunoprivileged cells and tissues, such as tumor cells to leak or lyse, undergo cell death, or otherwise cause or enhance an immune response to immunoprivileged cells and tissues, such as tumor cells.

i. Reduced toxicity

Microbes can be toxic to their hosts by manufacturing one or more compounds that worsen the health condition of the host. Toxicity to the host can be manifested in any of a variety of manners, including septic shock, neurological effects, or muscular effects. The microbes provided herein can have a reduced toxicity to the host. The reduced toxicity of a microbe of the present methods and compositions can range from a toxicity in which the host experiences no toxic effects, to a toxicity in which the host does not typically die from the toxic effects of the microbes. In some embodiments, the microbes are of a reduced toxicity such that a host typically has no significant long-term effect from the presence of the

microbes in the host, beyond any affect on tumorous, metastatic or necrotic organs or tissues. For example, the reduced toxicity can be a minor fever or minor infection, which lasts for less than about a month, and following the fever or infection, the host experiences no adverse effects resultant from the fever or infection. In another example, the reduced toxicity can be measured as an unintentional decline in body weight of about 5% or less for the host after administration of the microbes. In other examples, the microbe has no toxicity to the host.

Exemplary vaccinia viruses of the LIVP strain (a widely available attenuated) Lister strain) that have reduced toxicity compared to other vaccinia viruses employed and are further modified. Modified LIVP were prepared. These LIVP include insertions in the TK and HA genes and optionally in the locus designed F3. As an example of reduced toxicity, these recombinant vaccinia viruses were tested for their toxicity to mice with impaired immune systems (nude mice) relative to the corresponding wild type vaccinia virus. Intravenous (i.v.) injection of wild type vaccinia virus VGL (strain LIVP) at $1x10^7$ PFU/mouse causes toxicity in nude mice: three mice out of seven lost the weight and died (one mouse died in one week after virus injection, one mouse died ten days after virus injection). Recombinant vaccinia virus designated RVGL8 (LacZ inserted into F3 locus) did not show toxic effects in nude mice after i.v. injection of $1x10^7$ PFU/mouse. There were no readily detectable signs of RVGL8 virus-related toxicity. Therefore, insertion into NotI site (located in F3 gene) of vaccinia virus genome strain LIVP reduces toxicity of the vaccinia virus to the host. Similar modifications can be made to other pox viruses and other viruses to reduce toxicity thereof. Such modifications can be empirically identified, if necessary.

ii. Accumulate in immunoprivileged cells and tissues, such as tumor, not substantially in other organs

Microbes can accumulate in any of a variety of tissues and organs of the host. Accumulation can be evenly distributed over the entire host organism, or can be concentrated in one or a few organs or tissues, The microbes provided herein can accumulate in targeted tissues, such as immunoprivileged cells and tissues, such as

tumors and also metastases. In some embodiments, the microbes provided herein exhibit accumulation in immunoprivileged cells and tissues, such as tumor cells relative to normal organs or tissues that is equal to or greater than the accumulation that occurs with wild type microbes. In other embodiments the microbes provided herein exhibit accumulation in immunoprivileged cells and tissues, such as tumor cells that is equal to or greater than the accumulation in any other particular organ or tissue. For example, the microbes provided herein can demonstrate an accumulation in immunoprivileged cells and tissues, such as tumor cells that is at least about 2-fold greater, at least about 5-fold greater, at least about 10-fold greater, at least about 100-fold greater, at least about 1,000-fold greater, at least about 10,000-fold greater, at least about 100,000-fold greater, or at least about 1,000,000-fold greater, than the accumulation in any other particular organ or tissue.

In some embodiments, a microbe can accumulate in targeted tissues and cells, such as immunoprivileged cells and tissues, such as tumor cells, without accumulating in one or more selected tissues or organs. For example, a microbe can accumulate in tumor cells without accumulating in the brain. In another example, a microbe can accumulate in tumor cells without accumulating in neural cells. In another example, a microbe can accumulate in tumor cells without accumulating in ovaries. In another example, a microbe can accumulate in tumor cells without accumulating in the blood. In another example, a microbe can accumulate in tumor cells without accumulating in the heart. In another example, a microbe can accumulate in tumor cells without accumulating in the bladder. In another example, a microbe can accumulate in tumor cells without accumulating in testes. In another example, a microbe can accumulate in tumor cells without accumulating in the spleen. In another example, a microbe can accumulate in tumor cells without accumulating in the lungs.

One skilled in the art can determine the desired capability for the microbes to selectively accumulate in targeted tissue or cells, such as in a immunoprivileged cells and tissues, such as tumor rather than non-target organs or tissues, according to a variety of factors known in the art, including, but not limited to, toxicity of the

microbes, dosage, tumor to be treated, immunocompetence of host, and disease state of the host.

Provided herein as an example of selective accumulation in immunoprivileged cells and tissues, such as tumors relative to normal organs or tissues, presence of various vaccinia viruses was assayed in tumor samples and different organs. Wild type VGL virus was recovered from tumor, testes, bladder, and liver and as well as from brain. Recombinant virus RVGL8 was found mostly in tumors (in mouse #24, virus was found in testes, bladder and liver; in mouse #22 in testes), and no virus was recovered from brain tissue in six tested animals. Therefore, this finding demonstrates the tumor accumulation properties of a recombinant vaccinia virus of the LIVP strain with an insertion in the F3 gene for tumor therapy purposes.

iii. Ability to Elicit or Enhance Immune Response to Tumor Cells

The microorganisms herein cause or enhance an immune response to antigens in the targeted tissues or cells, such as immunoprivileged cells and tissues, such as tumor cells. The immune response can be triggered by any of a variety of mechanisms, including the presence of immunostimulatory cytokines and the release antigenic compounds that can cause an immune response.

Cells, in response to an infection such as a microorganismal infection, can send out signals to stimulate an immune response against the cells. Exemplary signals sent from such cells include antigens, cytokines and chemokines such as interferon-gamma and interleukin-15. The microorganism provided herein can cause targeted cells to send out such signals in response to infection by the microbes, resulting in a stimulation of the host's immune system against the targeted cells or tissues, such as tumor cells.

In another embodiment, targeted cells or tissues, such as tumor cells, can contain one or more compounds that can be recognized by the host's immune system in mounting an immune response against a tumor. Such antigenic compounds can be compounds on the cell surface or the tumor cell, and can be protein, carbohydrate, lipid, nucleic acid, or combinations thereof. Microbe-mediated

release of antigenic compounds can result in triggering the host's immune system to mount an immune response against the tumor. The amount of antigenic compound released by the tumor cells is any amount sufficient to trigger an immune response in a subject ; for example, the antigenic compounds released from one or more tumor cells can trigger a host immune response in the organism that is known to be accessible to leukocytes.

The time duration of antigen release is an amount of time sufficient for the host to establish an immune response to one or more tumor antigens. In some embodiments, the duration is an amount of time sufficient for the host to establish a sustained immune response to one or more tumor antigens. One skilled in the art can determine such a time duration based on a variety of factors affecting the time duration for a subject to develop an immune response, including the level of the tumor antigen in the subject, the number of different tumor antigens, the antigenicity of the antigen, the immunocompetence of the host, and the access of the antigenic material to the vasculature of the host. Typically, the duration of antigen release can be at least about a week, at least about 10 days, at least about two weeks, or at least about a month.

The microorganism provided herein can have any of a variety of properties that can cause target cells and tissues, such as tumor cells, to release antigenic compounds. Exemplary properties are the ability to lyse cells and the ability to elicit apoptosis in tumor cells. Microbes that are unable to lyse tumor cells or cause tumor cell death can nevertheless be used in the methods provided herein when such microbes can cause some release or display of antigenic compounds from tumor cells. A variety of mechanisms for antigen release or display without lysis or cell death are known in the art, and any such mechanism can be used by the microbes provided herein, including, but not limited to, secretion of antigenic compounds, enhanced cell membrane permeability, or altered cell surface expression or altered MHC presentation in tumor cells when the tumor cells can be accessed by the host's immune system. Regardless of the mechanism by which the host's immune system is activated, the net result of the presence of the microbes in the tumor is a

stimulation of the host's immune system, at least in part, against the tumor cells. In one example, the microbes can cause an immune response against tumor cells not infected by the microbes.

In one embodiment, the microbes provided herein can cause tumor cells to release an antigen that is not present on the tumor cell surface. Tumor cells can produce compounds such as proteins that can cause an immune response; however, in circumstances in which the antigenic compound is not on the tumor cell surface, the tumor can proliferate, and even metastasize, without the antigenic compound causing an immune response. Within the scope of the present methods, the microbes provided herein can cause antigenic compounds within the cell to release away from the cell and away from the tumor, which can result in triggering an immune response to such an antigen. Even if not all cells of a tumor are releasing antigens, the immune response can initially be targeted toward the "leaky" tumor cells, and the bystander effect of the immune response can result in further tumor cell death around the "leaky" tumor cells.

iv. Balance of Pathogenicity and Release of Tumor Antigens

Typical methods of involving treatment of targeted cells and tissues, such as immunoprivileged cells and tissues, such as tumors, are designed to cause rapid and complete removal thereof. For example, many viruses, bacterial or eukaryotic cells can cause lysis and/or apoptosis in a variety of cells, including tumor cells. Microorganisms that can vigorously lyse or cause cell death can be highly pathogenic, and can even kill the host. Furthermore, therapeutic methods based upon such rapid and complete lysis are typically therapeutically ineffective.

In contrast, the microorganisms provided herein are not aggressive in causing cell death or lysis. They can have only a limited or no ability to cause cell death as long as they accumulate in the target cells or tissues and result in alteration of cell membranes to cause leakage of antigens against which an immune response is mounted. It is desirable that their apoptotic or lytic effect is sufficiently slow or ineffective to permit sufficient antigenic leakage for a sufficient time for the host to mount an effective immune response against the target tissues. Such immune

response alone or in combination with the lytic/apoptotic effect of the microorganism results in elimination of the target tissue and also elimination of future development, such as metastases and reoccurrence, of such tissues or cells. While the microbes provided herein can have a limited ability to cause cell death, the microbes provided herein can nevertheless stimulate the host's immune system to attack tumor cells. As a result, such microorganisms also are typically unlikely to have substantial toxicity to the host.

In one embodiment, the microbes have a limited, or no, ability to cause tumor cell death, while still causing or enhancing an immune response against tumor cells. In one example, the rate of microorganism-mediated tumor cell death is less than the rate of tumor cell growth or replication. In another example, the rate of microorganism-mediated tumor cell death is slow enough for the host to establish a sustained immune response to one or more tumor antigens. Typically, the time for of cell death is sufficient to establish an anti-tumor immune response and can be at least about a week, at least about 10 days, at least about two weeks, or at least about a month, depending upon the host and the targeted cells or tissues.

In another embodiment, the microbes provided herein can cause cell death in tumor cells, without causing substantial cell death in non-tumor tissues. In such an embodiment, the microbes can aggressively kill tumor cells, as long as no substantial cell death occurs in non-tumor cells, and optionally, so long as the host has sufficient capability to mount an immune response against the tumor cells.

In one embodiment, the ability of the microbes to cause cell death is slower than the host's immune response against the microbes. The ability for the host to control infection by the microbes can be determined by the immune response (*e.g.*, antibody titer) against microorganismal antigens. Typically, after the host has mounted immune response against the microbes, the microbes can have reduced pathogenicity in the host. Thus, when the ability of the microbes to cause cell death is slower than the host's immune response against the microbes, microbe-mediated cell death can occur without risk of serious disease or death to the host. In one

example, the ability of the microbes to cause tumor cell death is slower than the host's immune response against the microbes.

b. Immunogenicity

The microorganisms provided herein also can be immunogenic. An immunogenic microorganism can create a host immune response against the microorganism. In one embodiment, the microorganisms can be sufficiently immunogenic to result in a large anti-(microorganism) antibody titer. The microorganisms provided herein can have the ability to elicit an immune response. The immune response can be activated in response to viral antigens or can be activated as a result of microorganismal-infection induced cytokine or chemokine production. Immune response against the microorganism can decrease the likelihood of pathogenicity toward the host organism.

Immune response against the microorganism also can result in target tissue or cell, such as tumor cell, killing. In one embodiment, the immune response against microorganismal infection can result in an immune response against tumor cells, including developing antibodies against tumor antigens. In one example, an immune response mounted against the microorganism can result in tumor cell killing by the "bystander effect," where uninfected tumor cells nearby infected tumor cells are killed at the same time as infected cells, or alternatively, where uninfected tumor cells nearby extracellular microorganisms are killed at the same time as the microorganisms. As a result of bystander effect tumor cell death, tumor cell antigens can be released from cells, and the host organism's immune system can mount an immune response against tumor cell antigens, resulting in an immune response against the tumor itself.

In one embodiment, the microorganism can be selected or modified to express one or more antigenic compounds, including superantigenic compounds. The antigenic compounds such as superantigens can be endogenous gene products or can be exogenous gene products. Superantigens, including toxoids, are known in the art and described elsewhere herein.

c. Replication Competent

The microorganisms provided herein can be replication competent. In a variety of viral or bacterial systems, the administered microorganism is rendered replication incompetent to limit pathogenicity risk to the host. While replication incompetence can protect the host from the microorganism, it also limits the ability of the microorganism to infect and kill tumor cells, and typically results in only a short-lived effect. In contrast, the microorganisms provided herein can be attenuated but replication competent, resulting in low toxicity to the host and accumulation mainly or solely in tumors. Thus, the microorganisms provided herein can be replication competent without creating a pathogenicity risk to the host.

Attenuation of the microorganisms provided herein can include, but is not limited to, reducing the replication competence of the microorganism. For example, a microorganism can be modified to decrease or eliminate an activity related to replication, such as a transcriptional activator that regulates replication in the microorganism. In an example, a microorganism, such as a virus, can have the viral thymidine kinase gene modified.

d. Genetic Variants

The microorganisms provided herein can be modified from their wild type form. Modifications can include any of a variety of changes, and typically include changes to the genome or nucleic acid molecules of the microorganisms. Exemplary nucleic acid molecular modifications include truncations, insertions, deletions and mutations. In an exemplary modification, a microorganismal gene can be modified by truncation, insertion, deletion or mutation. In an exemplary insertion, an exogenous gene can be inserted into the genome of the microorganism.

i. Modified Characteristics

Modifications of the microorganisms provided herein can result in a modification of microorganismal characteristics, including those provided herein such as pathogenicity, toxicity, ability to preferentially accumulate in tumor, ability to lyse cells or cause cell death, ability to elicit an immune response against tumor cells, immunogenicity, replication competence. Variants can be obtained by general

methods such as mutagenesis and passage in cell or tissue culture and selection of desired properties, as is known in the art, as exemplified for respiratory syncytial virus in Murphy *et al.*, Virus Res. 1994, 32:13-26.

Variants also can be obtained by mutagenic methods in which nucleic acid residues of the microorganism are added, removed or modified relative to the wild type. Any of a variety of known mutagenic methods can be used, including recombination-based methods, restriction endonuclease-based methods, and PCR-based methods. Mutagenic methods can be directed against particular nucleotide sequences such as genes, or can be random, where selection methods based on desired characteristics can be used to select mutated microorganisms. Any of a variety of microorganismal modifications can be made, according to the selected microorganism and the particular known modifications of the selected microorganism.

ii. Exogenous Gene Expression

The microorganisms provided herein also can have the ability to express one or more exogenous genes. Gene expression can include expression of a protein encoded by a gene and/or expression of an RNA molecule encoded by a gene. In some embodiments, the microorganisms can express exogenous genes at levels high enough that permit harvesting products of the exogenous genes from the tumor. Expression of endogenous genes can be controlled by a constitutive promoter, or by an inducible promoter. Expression can also be influenced by one or more proteins or RNA molecules expressed by the microorganism. An exemplary inducible promoter system can include a chimeric transcription factor containing a progesterone receptor fused to the yeast GAL4 DNA-binding domain and to the activation domain of the herpes simplex virus protein VP16, and a synthetic promoter containing a series of GAL4 recognition sequences upstream of the adenovirus major late E1B TATA box, linked to one or more exogenous genes; in this exemplary system, administration of RU486 to a subject can result in induction of the exogenous genes. Exogenous genes expressed can include genes encoding a therapeutic gene product, genes encoding a detectable gene product such as a gene

product that can be used for imaging, genes encoding a gene product to be harvested, genes encoding an antigen of an antibody to be harvested. The microorganisms provided herein can be used for expressing genes in vivo and in vitro. Exemplary proteins include reporter proteins (E. coli β-galactosidase, β-glucuronidase, xanthineguanine phosphoribosyltransferase), proteins facilitating detection, *i.e.*, a detectable protein or a protein capable of inducing a detectable signal, (*e.g.*, luciferase, green and red fluorescent proteins, transferrin receptor), proteins useful for tumor therapy (pseudomonas A endotoxin, diphtheria toxin, p53, Arf, Bax, tumor necrosis factor-alpha, HSV TK, E. coli purine nucleoside phosphorylase, angiostatin, endostatin, different cytokines) and many other proteins.

iii. Detectable gene product

The microorganisms provided herein can express one or more genes whose products are detectable or whose products can provide a detectable signal. A variety of detectable gene products, such as detectable proteins are known in the art, and can be used with the microorganisms provided herein. Detectable proteins include receptors or other proteins that can specifically bind a detectable compound, proteins that can emit a detectable signal such as a fluorescence signal, enzymes that can catalyze a detectable reaction or catalyze formation of a detectable product.

In some embodiments, the microorganism expresses a gene encoding a protein that can emit a detectable signal or that can catalyze a detectable reaction. A variety of DNA sequences encoding proteins that can emit a detectable signal or that can catalyze a detectable reaction, such as luminescent or fluorescent proteins, are known and can be used in the microorganisms and methods provided herein. Exemplary genes encoding light-emitting proteins include genes from bacterial luciferase from Vibrio harveyi (Belas etal., Science 218 (1982), 791-793), bacterial luciferase from Vibrio fischerii (Foran and Brown, Nucleic acids Res. 16 (1988), 177), firefly luciferase (de Wet *et al.* , Mol. Cell. Biol. 7(1987), 725-737), aequorin from Aequorea victoria (Prasher *et al.* , Biochem. 26 (1987), 1326-1332), Renilla luciferase from Renilla renformis (Lorenz *et al.* , PNAS USA 88 (1991), 4438-4442) and green fluorescent protein from Aequorea victoria (Prasher *et al.* , Gene 111

(1987), 229-233). Transformation and expression of these genes in microorganisms can permit detection of microorganismal colonies, for example, using a low light imaging camera. Fusion of the lux Aand lux B genes can result in a fully functional luciferase protein (Escher *et al.*, PNAS 86 (1989), 6528-6532). This fusion gene (Fab2) has introduced into a variety of microorganisms followed by microorganismal infection and imaging based on luciferase expression. In some embodiments, luciferases expressed in bacteria can require exogenously added substrates such as decanal or coelenterazine for light emission. In other embodiments, microorganisms can express a complete lux operon, which can include proteins that can provide luciferase substrates such as decanal. For example, bacteria containing the complete lux operon sequence, when injected intraperitoneally, intramuscularly, or intravenously, allowed the visualization and localization of bacteria in live mice indicating that the luciferase light emission can penetrate the tissues and can be detected externally (Contag *et al.*, Mol. Microbiol. 18 (1995), 593-603).

In other embodiments, the microorganism can express a gene that can bind a detectable compound or that can form a product that can bind a detectable compound. A variety of gene products, such as proteins, that can specifically bind a detectable compound are known in the art, including receptors, metal binding proteins, ligand binding proteins, and antibodies. Any of a variety of detectable compounds can be used, and can be imaged by any of a variety of known imaging methods. Exemplary compounds include receptor ligands and antigens for antibodies. The ligand can be labeled according to the imaging method to be used. Exemplary imaging methods include any of a variety magnetic resonance methods such as magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), and also include any of a variety of tomographic methods including computed tomography (CT), computed axial tomography (CAT), electron beam computed tomography (EBCT), high resolution computed tomography (HRCT), hypocycloidal tomography, positron emission tomography (PET), single-photon

emission computed tomography (SPECT), spiral computed tomography and ultrasonic tomography.

Labels appropriate for magnetic resonance imaging are known in the art, and include, for example, gadolinium chelates and iron oxides. Use of chelates in contrast agents is known in the art. Labels appropriate for tomographic imaging methods are known in the art, and include, for example, β-emitters such as ^{11}C, ^{13}N, $^{15}0$ or ^{64}Cu or (b) γ-emitters such as^{123}I. Other exemplary radionuclides that can, be used, for example, as tracers for PET include ^{55}Co, ^{67}Ga, ^{68}Ga, $^{60}Cu(II)$, $^{67}Cu(II)$, ^{57}Ni, ^{52}Fe and ^{18}F. Examples of useful radionuclide-labeled agents are ^{64}Cu-labeled engineered antibody fragment (Wu *et al.*, PNAS USA 97 (2002), 8495-8500), ^{64}Cu-labeled somatostatin (Lewis *et al.*, J. Med. Chem. 42(1999), 1341-1347), ^{64}Cu-pyruvaldehyde-bis (N4methylthiosemicarbazone)(64Cu-PTSM) (Adonai *et al.*, PNAS USA 99 (2002), 3030-3035), ^{52}Fe-citrate (Leenders *et al.*, J. Neural.Transm.Suppl. 43 (1994), 123-132), $^{52}Fe/^{52m}Mn$-citrate (Calonder *et al.*, J. Neurochem. 73 (1999), 2047-2055) and ^{52}Fe-labeled iron (III) hydroxide-sucrose complex (Beshara *et al.*, Br. J. Haematol. 104 (1999), 288-295,296-302).

iv. Therapeutic gene product

The microorganisms provided herein can express one or more genes whose products cause cell death or whose products cause an anti-tumor immune response, such genes can be considered therapeutic genes. A variety of therapeutic gene products, such as toxic or apoptotic proteins, or siRNA, are known in the art, and can be used with the microorganisms provided herein. The therapeutic genes can act by directly killing the host cell, for example, as a channel-forming or other lytic protein, or by triggering apoptosis, or by inhibiting essential cellular processes, or by triggering an immune response against the cell, or by interacting with a compound that has a similar effect, for example, by converting an less active compound to a cytotoxic compound.

In some embodiments, the microorganism can express a therapeutic protein. A large number of therapeutic proteins that can be expressed for tumor treatment are known in the art, including, but not limited to tumor suppressors, toxins, cytostatic

proteins, and cytokines. An exemplary, non-limiting list of such proteins includes WT1, p53, p16, Rb, BRCA1, cystic fibrosis transmembrane regulator (CFTR), Factor VIII, low density lipoprotein receptor, beta-galactosidase, alpha-galactosidase, beta-glucocerebrosidase, insulin, parathyroid hormone, alpha-1-antitrypsin, rsCD40L, Fas-ligand, TRAIL, TNF, antibodies, microcin E492, diphtheria toxin, Pseudomonas exotoxin, Escherichia coli Shig toxin, Escherichia coli Verotoxin 1, and hyperforin.

In other embodiments, the microorganism can express a protein that converts a less active compound into a compound that causes tumor cell death. Exemplary methods of conversion of such a prodrug compound include enzymatic conversion and photolytic conversion. A large variety of protein/compound pairs are known in the art, and include, but are not limited to Herpes simplex virus thymidine kinase/gancyclovir, varicella zoster thymidine kinase/gancyclovir, cytosine deaminase/5-fluorouracil, purine nucleoside phosphorylase/6-methylpurine deoxyriboside, beta lactamase/cephalosporin-doxorubicin, carboxypeptidase G2/4-[(2-chloroethyl)(2-mesuloxyethyl)amino]benzoyl-L-glutamic acid, cytochrome P450/acetominophen, horseradish peroxidase/indole-3-acetic acid, nitroreductase/CB1954, rabbit carboxylesterase/7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, mushroom tyrosinase/bis-(2-chloroethyl)amino-4-hydroxyphenylaminomethanone 28, beta galactosidase/1-chloromethyl-5-hydroxy-1,2-dihyro-3H-benz[e]indole, beta glucuronidase/epirubicin-glucuronide, thymidine phosphorylase/5'-deoxy5-fluorouridine, deoxycytidine kinase/cytosine arabinoside, and linamerase/linamarin.

In another embodiment, the therapeutic gene product can be an siRNA molecule. The siRNA molecule can be directed against expression of a tumor-promoting gene, such as, but not limited to, an oncogene, growth factor, angiogenesis promoting gene, or a receptor. The siRNA molecule also can be directed against expression of any gene essential for cell growth, cell replication or cell survival. The siRNA molecule also can be directed against expression of any gene that stabilizes the cell membrane or otherwise limits the number of tumor cell

antigens released from the tumor cell. Design of an siRNA can be readily determined according to the selected target of the siRNA; methods of siRNA design and downregulation of genes are known in the art, as exemplified in U.S. Pat. Pub. No. 20030198627.

In one embodiment, the therapeutic compound can be controlled by a regulatory sequence. Suitable regulatory sequences which, for example, are functional in a mammalian host cell are well known in the art. In one example, the regulatory sequence can contain a natural or synthetic vaccinia virus promoter. In another embodiment, the regulatory sequence contains a poxvirus promoter. When viral microorganisms are used, strong late promoters can be used to achieve high levels of expression of the foreign genes. Early and intermediate-stage promoters, however, can also be used. In one embodiment, the promoters contain early and late promoter elements, for example, the vaccinia virus early/late promoter p7.5, vaccinia late promoter p11, a synthetic early/late vaccinia pE/L promoter (Patel *et al.*, (1988), Proc. Natl. Acad. Sci. USA 85, 9431-9435; Davison and Moss, (1989), J Mol Biol 210, 749-769; Davison *et al.*, (1990), Nucleic Acids Res. 18, 4285-4286; Chakrabarti *et al.*, (1997), BioTechniques 23, 1094-1097).

v. Expressing a superantigen

The microorganisms provided herein can be modified to express one or more superantigens. Superantigens are antigens that can activate a large immune response, often brought about by a large response of T cells. A variety of superantigens are known in the art including, but not limited to, diphtheria toxin, staphylococcal enterotoxins (SEA, SEB, SEC1, SEC2, SED, SEE and SEH), Toxic Shock Syndrome Toxin 1, Exfoliating Toxins (EXft), Streptococcal Pyrogenic Exotoxin A, B and C (SPE A, B and C), Mouse Mammary Tumor Virus proteins (MMTV), Streptococcal M proteins, Clostridial Perfringens Enterotoxin (CPET), mycoplasma arthritis superantigens.

Since many superantigens also are toxins, if expression of a microorganism of reduced toxicity is desired, the superantigen can be modified to retain at least some of its superantigenicity while reducing its toxicity, resulting in a compound

such as a toxoid. A variety of recombinant superantigens and toxoids of superantigens are known in the art, and can readily be expressed in the microorganisms provided herein. Exemplary toxoids include toxoids of diphtheria toxin, as exemplified in U.S. Pat. No. 6,455,673 and toxoids of Staphylococcal enterotoxins, as exemplified in U.S. Pat. Pub. No. 20030009015.

vi. Expressing a gene product to be harvested

Exemplary genes expressible by a microorganism for the purpose of harvesting include human genes. An exemplary list of genes includes the list of human genes and genetic disorders authored and edited by Dr. Victor A. McKusick and his colleagues at Johns Hopkins University and elsewhere, and developed for the World Wide Web by NCBI, the National Center for Biotechnology Information. Online Mendelian Inheritance in Man, OMIM™. Center for Medical Genetics, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), 1999. and those available in public databases, such as pubmed and genbank (see, *e.g.*, (ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) These genes include, but are not limited to: 239f2h9, 3pk, 4ebp1, 4ebp2, al1, al2m1, al2m2, al2m3, al2m4, al5, a1b, albg, alst, a2m, a2mr, a2mrap, aa, aaa, aaa, aabt, aac1, aac2, aact, aadac, aanat, aars, aas, aat, aavs1, abc1, abc2, abc3, abc7, abc8, abcr, abi1, abl1, abl2, abl1, abo, abp, abp1, abpa, abpx, abr, acaa, acac, acaca, acacb, acad1, acadm, acads, acadsb, acadv1, acat, acat1, acat2, acc, accb, accn1, accn2, accpn, ace1, ach, ache, achm1, achm2, achrb, achrd, achrg, acls, acly, aco1, aco2, acox, acox1, acox2, acox3, acp1, acp2, acp5, acpp, acr, acrv1, acs3, acs3, acs4, act2, act35, acta1, acta2, acta3, actb, actc, actg1, actg2, act11, actn1, actn2, actn3, actsa, acug, acvr1, acvr2b, acvrl1, acvrlk1, acvrlk2, acvrlk3, acy1, ad1, ad2, ad3, ad4, ad5, ada, adam10, adam11, adam12, adam3, adam3a, adam3b, adam8, adar, adarb1, adarb2, adcp1, adcp2, adcy1, adcy2, adcy3, adcy3, adcy4, adcy5, adcy6, adcy7, adcy8, adcy9, adcyap1, adcyaplr1, add1, add2, add3, add1, adfn, adh1, adh2, adh3, adh4, adh5, adh7, adhaps, adhc1@, adhr, adhr, adk, ad1, adm, admlx, adora1, adora2a, adora2b, adora21, adora21, adora3, adprt, adra1a, adra1b, adra1c, adra1d, adra2a, adra2b,

adra2c, adra211, adra212, adra2r, adrb1, adrb1r, adrb2, adrb2rl1, adrb3, adrbk1, adrbk2, ads1, adss, adtb1, adx, adxr, ae1, ae2, ae3, aegl1, aemk, aes, af10, af17, af4, af6, af8t, af9, afd1, afdn, afg3, afg311 , afm, afp, afx1, aga, agc1, ager, ag1, agmx1, agmx2, agp1, agp7, agps, agrn, agrp, agrt, ags, agt, agti1, agtr1, agtr1a, agtr2, agtrl1, agxt, ahc, ahcy, ahd, ahds, ahnak, aho2, ahr, ahsg, ahx, aib1, aic, aic1, aied, aih1, aih2, aih3, aim1, air, airc, aire, ak1, ak2, ak3, akap149, akt1, akt2, aku, alad, alas1, alas2, alb, alb2, alba, alcam, ald, aldh1, aldh10, aldh2, aldh3, aldh4, aldh5, aldh6, aldh9, ald11, aldoa, aldob, aldoc, aldr1, alds, alk, alk1, alk2, alk3, alk6, alms1, alox12, alox15, alox5, alp, alpi, alp1, alpp, alpp12, alr, alr, als1, als2, als4, als5, alss, ambn, ambp, amcd1, amcd2b, amcn, amcn1, amcx1, amd1, amdm, amelx, amely, amfr, amg, amg1, amgx, amh, amhr, amhr2, aml1, aml1t1, aml2, aml3, amog, ampd1, ampd2, ampd3, amph, amph1, ampk, amt, amy1a, amy1b, amy1c, amy2a, amy2b, an2, anc, ancr, ang, ang1, anh1, ank1, ank2, ank3, anop1, anova, anp, anpep, anpra, anprb, anprc, ans, ant1, ant2, ant3, ant3y, anx1, anx11, anx13, anx2, anx214, anx3, anx4, anx5, anx6, anx7, anx8, aoah, aoc2, aox1, ap2tf, apah1, apba1, apba2, apbb1, apbb2, apc, apcs, ape, apeced, apeh, apex, api1, api2, api3, apj, aplp, aplp1, aplp2, apnh, apo31, apoa1, apoa2, apoa4, apob, apobec1, apoc1, apoc2, apoc3, apoc4, apod, apoe, apoer2, apoh, apolmt, apolp1@, apolp2@, app, appbp1, appl1, aprf, aprt, aps, apt1, aptl1g1, apx1, apy, aqdq, aqp0, aqp1, aqp2, aqp21, aqp3, aqp4, aqp5, aqp6, aqp7, ar, ar1, ara, araf1, araf2, arcn1, ard1, ard1, areg, arf1, arf2, arf3, arf41, arf5, arg, arg1, args, arh12, arh6, arh9, arha, arhb, arhc, arhg, arhgap2, arhgap3, arhgap6, arhgdia, arhgdib, arhh, arix, arl2, armd1, arnt, arnt1, aro, arp, arp1, arpkd, arr3, arrb1, arrb2, arsa, arsacs, arsb, arsc1, arsc2, arsd, arse, arsf, art, art1, art3, art4, arts, arvd1, arvd2, arvd3, arvd4, as, asat, asb, ascl1, ascl2, asct1, asd1, asd2, asgr1, asgr2, ash1, asip, as1, asln, asm1, asma, asmd, asmt, asmtlx, asmty, asnrs, asns, aspa, ass, astm1, astn, asv, at, at1, at2r1, at3, ata, atbf1, atcay, atf1, ath1, aths, atm, atoh1, atox1, atp1a1, atp1a2, atp1a3, atp1a11, atp1b1, atp1b2, atp1b3, atp1b11, atp1g1, atp2a1, atp2a2, atp2a3, atp2b, atp2b1, atp2b2, atp2b2, atp2b3, atp2b4, atp4a, atp4b, atp5, atp5a, atp5b, atp5g1, atp5g2, atp5g3, atp5o, atp6a, atp6b1, atp6c, atp6e, atp6n1, atp7a, atp7b, atpm, atpsb, atpsk1, atpsk2, atq1,

atr, atr, atr1, atr1, atr2, atrc1, atrc2, atrx, ats, atsv, atx1, atx2, au, auf1, auf1a, aut, avcd, aved, avp, avpr1a, avpr1b, avpr2, avpr3, avrp, avsd, awa1, ax1, ax11g, axsf, azf1, azf2, azgp1, azu1, b120, b144, blg1, b29, b2m, b2mr, b3galt4, b4galt1, ba2r, bab1, bag1, bai1, bai2, bai3, bak1, bam22, bap1, bap135, bapx1, bard1, bark2, bas, bat1, bat2, bat3, bat4, bat5, bax, bb1, bbbg1, bbbg2, bbs1, bbs2, bbs3, bbs4, bbs5, bcas1, bcat1, bcat2, bcate2, bcd1, bcei, bche, bckdha, bckdhb, bcl1, bcl10, bcl2, bcl2a1, bcl212, bcl3, bcl5, bcl6, bcl7, bcl7a, bcl8, bcl9, bclw, bcm, bcm1, bcma, bcns, bcns, bcp, bcpm, bcpr, bcr, bcrl2, bcrl3, bcrl4, bcsg1, bct1, bct2, bdb, bdb1, bdc, bde, bdkrb1, bdkrb2, bdmf, bdmr, bdnf, bed, bedp, bek, bene, bevi, bf, bf1, bf2, bfhd, bfic, bfls, bfnc2, bfp, bfsp1, bft, bglap, bgmr, bgn, bgp, bhd, bhpcdh, bhr1, bicd1, bid, bigh3, bin1, bir, bjs, bkma1, blast1, blau, blk, blm, blmh, bltr, blvra, blvrb, blym, bmal1, bmd, bmh, bmi1, bmp1, bmp2, bmp2a, bmp2b1, bmp3, bmp4, bmp5, bmp6, bmp7, bmp8, bmpr1a, bmpr1b, bmx, bmyb, bn51t, bnc, bnc1, bnp, bor, bpad, bpag1, bpag2, bpes, bpes1, bpes2, bpgm, bph1, bpi, br, br140, braf, brca1, brca2, brca3, brcacox, brcd1, brcd2, brdt, brf1, brhc, bric, brks, brn3a, brn3b, brn3c, brrn1, brw1c, bs, bsap, bsep, bsf2, bsg, bsnd, bss1, bst1, bst2, btak, btc, btd, bteb, bteb1, btg1, btg2, bths, btk, btk1, btn, bts, bub1b, bubr1, bwr1a, bwr1b, bws, bwscr1a, bwscr1b, bzrp, bzx, c11orf13, c1nh, c1qa, c1qb, c1qbp, c1qg, c1r, c1s, c2, c21orf1, c21orf2, c21orf3, c2ta, c3, c3br, c3dr, c3g, c4a, c4b, c4bpa, c4bpb, c4f, c4s, c5, c5ar, c5r1, c6, c7, c8a, c8b, c8g, c9, ca1, ca12, ca125, ca2, ca21h, ca3, ca4, ca5, ca6, ca7, ca8, ca9, caaf1, cabp9k, cac, cac@, caca, cacd, cacna1a, cacna1b, cacna1c, cacna1d, cacna1e, cacna1f, cacna1s, cacna2, cacnb1, cacnb2, cacnb3, cacnb4, cacng, cacnl1a1, cacnl1a2, cacnl1a3, cacnl1a4, cacnl1a5, cacnl1a6, cacnl2a, cacnlb1, cacnlg, cacp, cact, cacy, cad, cad11, cadasi1, cae1, cae3, caf, caf1a, caga, cagb, cain, cak, cak1, cal11, calb1, calb2, calb3, calc1, calc2, calca, calcb, calcr, cald1, calla, calm1, calm2, calm3, calm11, calm13, calna, calna3, calnb, calnb1, calr, cals, calt, calu, cam, camk4, camkg, caml1, camlg, camp, can, canp3, canx, cap2, cap3, cap37, capb, capg, cap1, capn1, capn2, capn3, capn4, cappa2, cappb, capr, caps, capza2, capzb, car, carp, cars, cart1, cas, cas2, casi1, casp1, casp10, casp2, casp3, casp3, casp4, casp5, casp6, casp7, casp8, casq1, casq2, casr cast, cat, cat1, cat4, catf1,

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ttpa, ttr, tuba3, tubal1, tubb, tufm, tuft1, tulp1, tuple1, tw, tweak, twik1, twist, txgp11, txk, txn, txnr, txnrd1, tyh, tyk1, tyk2, tyk3, tyms, tyr, tyr1, tyro3, tyrp1, tyrp2, tys, u17hg, ulrnp, u22hg, u2af1, u2aflrs1, u2aflrs2, u2aflrs3, uba52, ubb, ubc, ubc4, ubc7, ubc8, ubch2, ubc1, ube1, ube2, ube2a, ube2b, ube2e2, ube2g, ube2g2, ube2h, ube2i, ube211, ube2v1, ube3a, ubh1, ubid4, ub11, uch11, ucn, ucp1, ucp2, ucp3, udpgdh, uev1, ufd11, ufs, ugalt, ugb, ugcg, ugdh, ugn, ugp1, ugp2, ugpp2, ugt1, ugt1a1, ugt2b11, ugt2b15, ugt2b17, ugt2b4, ugt2b7, ugt2b8, ugt2b9, ugt1, uhg, uhx1, ukhc, umod, umph2, umpk, umps, unc18, unc18b, und, ung, unr, unr, uox, up, upk1b, ups, uqbp, uqcrb, uqcrc1, uqcrc2, uqcrfs1, uqor1, uqor13, uqor22, urk, urkr, uroc, urod, uros, usf1, usf2, ush1, ush1a, ush1b, ush1c, ush1d, ush1e, ush1f, ush2a, ush3, usp11, usp5, usp7, usp9x, usp9y, ut1, ut2, ute, utr, utrn, utx, uty, uv20, uv24, uvo, vacht, vacm1, vamp1, vamp2, vars1, vasp, vat1, vat2, vav, vav1, vav2, vbch, vbp1, vcam1, vcf, vc1, vcp, vdac1, vdac2, vdd1, vdi, vdr, vegf, vegfb, vegfd, vegfr3, vgf, vg1, vgr1, vh1, vhr, vil1, vil2, vim, vip, vipr1, vipr2, vis1, vla1, vla5a, vlacs, vlcad, vldlr, vmat1, vmcm, vmd1, vmd2, vnra, vnt, vp, vpp1, vpp3, vpreb1, vpreb2, vrf, vrk1, vrk2, vrnf, vrni, vsn11, vtn, vwf, vws, waf1, wars, was, wbs, wd1, wdr2, wee1, wfrs, wfs, wfs1, wgn1, whcr, wi, wisp1, wisp2, wisp3, wnd, wnt1, wnt10b, wnt13, wnt14, wnt15, wnt2, wnt3, wnt5a, wnt7a, wnt7b, wnt8b, wrb, wrn, ws1, ws2a, ws2b, ws4, wsn, wss, wss, wt1, wt2, wt3, wt4, wt5, wts, wts1, wws, x11, xbp1, xbp2, xce, xdh, xe169, xe7, xe7y, xg, xgr, xh2, xiap, xic, xist, xk, xla, xla2, xlp, xlpd, xlrs1, xm, xpa, xpb, xpc, xpcc, xpct, xpf, xpf, xpg, xpmc2h, xpnpep2, xpo1, xrcc1, xrcc2, xrcc3, xrcc4, xrcc5, xrcc9, xrs, xs, xwnt2, yb1, yes1, yk140, y11, yrrm1, yt, ywha1, ywhab, ywhah, ywhaz, yy1, zac, zag, zan, zap70, zf87, zfm1, zfp3, zfp36, zfp37, zfx, zfy, zic1, zic2, zic3, zipk, znf1, znf10, znf117, znf11a, znf11b, znf12, znf121, znf123, znf124, znf125, znf126, znf13, znf14, znf141, znf144, znf146, znf147, znf157, znf16, znf160, znf162, znf163, znf165, znf169, znf173, znf179, znf189, znf19, znf192, znf193, znf195, znf198, znf2, znf20, znf200, znf204, znf217, znf22, znf23, znf24, znf25, znf26, znf27, znf29, znf3, znf32, znf34, znf35, znf36, znf38, znf4, znf40, znf41, znf42, znf44, znf45, znf46, znf5, znf6, znf69, znf7, znf70, znf71, znf72, znf73, znf74, znf75, znf75a, znf75c, znf76, znf77,

znf79, znf8, zn80, znf81, znf83, znf9, znfc150, znfc25, znfxy, znt3, znt4, zp3a, zp3b, zpk, zws1, and zyx.

Furthermore, genes from bacteria, plants, yeast, and mammals (*e.g.*, mice) can be used with the microorganisms provided herein. Non-limiting examples of E. coli genes include: aarF, aas, aat, abpS, abs, accA, accB, accC, accD, acd, aceA, aceB, aceE, aceF, aceK, ackA, ackB, acnA, acnB, acpD, acpP, acpS, acpX, acrA, acrB, acrC, acrD, acrE, acrF, acrR, acs, ada, add, adhB, adhC, adhE, adhR, adiA, adiY, adk, aegA, aer, aes, agaA, agaB, agaC, agaD, agaI, agaR, agaS, agaV, agaW, agaZ, agp, ahpC, ahpF, aidB, ais, alaS, alaT, alaU, alaV, alaW, alaX, aldA, aldB, aldH, alkA, alkB, alpA, alr, alsA, alsB, alsC, alsE, alsK, alx, amiA, amiB, amn, ampC, ampD, ampE, ampG, ampH, amtB, amyA, ansA, ansB, apaG, apaH, aphA, appA, appB, appC, appY, apt, aqpZ, araA, araB, araC, araD, araE, araF, araG, araH, araJ, arcA, arcB, argA, argB, argC, argD, argE, argF, argG, argH, argI, argM, argP, argQ, argR, argS, argT, argU, argV, argW, argX, argY, argZ, aroA, aroB, aroC, aroD, aroE, aroF, aroG, aroH, aroI, aroK, aroL, aroM, aroP, aroT, arsB, arsC, arsR, artI, artJ, artM, artP, artQ, ascB, ascF, ascG, asd, aslA, aslB, asmA, asnA, asnB, asnC, asnS, asnT, asnU, asnV, asnW, aspA, aspC, aspS, aspT, aspU, aspV, asr, asu, atoA, atoB, atoC, atoD, atoS, atpA, atpB, atpC, atpD, atpE, atpF, atpG, atpH, atpI, avtA, azaA, azaB, azl, bacA, baeR, baeS, barA, basR, basS, bax, bcp, bcr, betA, betB, betI, betT, bfd, bfm, bfr, bglA, bglB, bglF, bglG, bglJ, bglT, bglX, bioA, bioB, bioC, bioD, bioF, bioH, bioP, bipA, birA, bisC, bisZ, blc, bolA, bRNQ, brnR, brnS brnT, btuB, btuc, btuD, btuE, btuR, bymA, cadA, cadB, cadC, cafA, caiA, caiB, caiC, caiD, caiE, caiF, caiT, calA, caiC, calD, can, carA, carB, cbl, cbpA, cbt, cca, ccmA, ccmB, ccmC, ccmD, ccmE, ccmF, ccmG, ccmH, cdd, cde, cdh, cdsA, cdsS, cedA, celA, celB, celC, celD, celF, cfa, cfcA, chaA, chaB, chaC, cheA, cheB, cheR, cheW, cheY, cheZ, chpA, chpB, chpR, chpS, cirA, citA, citB, cld, cipA, clpB, clpP, clpX, cls, cmk, cmlA, cmr, cmtA, cmtB, coaA, cobS, cobT, cobU, codA, codB, cof, cog?, corA, cpdA, cpdB, cpsA, cpsB, cpsC, cpsD, cpsE, cpsF, cpsG, cpxA, cpxB, cpxP, cpxR, crcA, crcB, creA, creB, creC, creD, crg, crl, crp, crr, csdA, csgA, csgB, csgD, csgE, csgF, csgG, csiA, csiB, csiC, csiD, csiE, csiF, cspA, cspB, cspC, cspD,

cspE, cspG, csrA, csrB, cstA, cstC, cup, cutA, cutC, cutE, cutF, cvaA(ColV), cvaB(ColV), cvaC(Co-IV), cvi(ColV), cvpA, cxm, cyaA, cybB, cybC, cycA, cydA, cydB, cydC, cydD, cynR, cynS, cynT, cynX, cyoA, cyoB, cyoC, cyoD, cyoE, cysA, cysB, cysC, cysD, cysE, cysG, cysH, cysI, cysJ, cysK, cysM, cysN, cysP, cysQ, cysS, cysT, cysU, cysW, cysX?, cysZ?, cytR, dacA, dacB, dacC, dacD, dadA, dadB, dadQ, dadX, dam, dapA, dapB, dapD, dapE, dapF, dbpA, dcd, dcm, dcp, dcrB, dctA, dctB, dcuA, dcuB, dcuC, ddlA, ddlB, ddpA, ddpB, ddpC, ddpD, ddpF, ddpX, deaD, dedA, dedD, def, degP, degQ, degS, del, deoA, deoB, deoC, deoD, deoR, dfp, dgd, dgkA, dgkR, dgoA, dgoD, dgoK, dgoR, dgoT, dgsA, dgt, dicA, dicB, dicC, dicF, dinB, dinD, dinF, dinG, dinI, dinY, dipZ, djlA, dksA, dld, dmsA, dmsB, dmsC, dnaA, dnaB, dnaC, dnaE, dnaG, dnaI, dnaJ, dnaK, dnaL, dnaN, dnaQ, dnaT, dnaX, dppA, dppB, dppC, dppD, dppF, dppG, dps, dsbA, dsbB, dsbC, dsbG, dsdA, dsdC, dsdX, dsrA, dsrB, dut, dvl, dxs, ebgA, ebgB, ebgC, ebgR, ecfa, eco, ecpD, eda, edd, efp, enirA, emrB, emrD, emrE, endA, eno, entA, entB, entC, entD, entE, entF, envN envP, envQ, envR, envT, envY, envZ, epd, EppA, minigene, EppB, minigene, EppC, minigene, EppD, minigene, EppE, minigene, EppG, minigene, EppH, minigene, era, esp, evgA, evgS, exbB, exbC, exbD, expA, exuR, exuT, fabA, fabB, fabD, fabF, fabG, fabH, fabI, fabZ, fadA, fadB, fadD, fadE, fadH, fadL, fadR, farR, fatA, fbaA, fbaB, fbp, fcl, fcsA, fdhD, fdhE, fdhF, fdnG, fdnH, fdnI, fdoG, fdoH, fdoI, fdrA, fdx, feaB, feaR, fecA, fecB, fecC, fecD, fecE, fecI, fecR, feoA, feoB, fepA, fepB, fepC, fepD, fepE, fepG, fes, fexB, ffh, ffs, fhlA, fhlB, fhuA , fhuB, fhuD, fhuE, fhuF, fic, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, fipB, fipC, fis, fiu, fixA, fixB, fixC, fixX, fklB, fkpA, fldA, flgA, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, flgL, flgM, flgN, flhA, flhB, flhc, flhD, fliA, fliC, fliD, fliE, fliF, fliG, fliH, fliI, fliJ, fliK, fliL, fliM, fliN, fliO, flip, fliQ, fliR, fliS, fliT, fliY, fliZ, flk, flu, fmt, fnr, focA, focB, folA, folC, folD, folE, folK, folP, folX, fpr, frdA, frdB, frdC, frdD, frr, fruA, fruB, fruK, fruR, fsr, ftn, ftsA, ftsE, ftsI, ftsJ, ftsK, ftsL, ftsN, ftsQ, ftsW, ftsX, ftsY, ftsZ, fucA, fucI, fucK, fucO, fucP, fucR, fumA, fumB, fumC, fur, fusA, fusB, gabC gabD, gabP, gabT, gadA, gadB, gadR, galE, galF, galK, galM, galP, galR, galS, galT, galU, gapA, gapC, garA, garB, gatA,

gatB, gatC, gatD, gatR, gatY, gatZ, gcd, gcl, gcpE, gcvA, gcvH, gcvP, gcvR, gcvT, gdhA, gef, ggt, gidA, gidB, gip, glcB, glcC, glcD, glcE, glcG, gldA, glf, glgA, glgB, glgC, glgP, glgS, glgX, glk, glmM, glmS, glmU, glmX, glnA, glnB, glnD, glnE, glnG, glnH, glnK, glhL, glnP, glnQ, glnR, glnS, glnT, glnU, glnV, glnW, glnX, gloA, glpA, glpB, glpC, glpD, glpE, glpF, glpG, glpK, glpQ, glpR, glpT, glpX, gltA, gltB, gltD, gltE, gltF, gltH, gltJ, gltK, gltL, gltM, gltP, gltR, gltS, gltT, gltU, gltv, gltW, gltX, glyA, glyQ, glyS, glyT, glyU, glyv, glyW, glyX, glyY, gmd, gmk, gmm, gnd, gntK, gntP, gntR, gntS, gntT, gntU, gntV, goaG, gor, gph, gpmA, gpp, gprA, gprB, gpsA, gpt, greA, greB, groL, groS, grpE, grxA, grxB, grxC, gshA, gshB, gsk, gsp, gsp*, gst, guaA, guaB, guaC, gurB, gurC, gutM, gutQ, gyrA, gyrB, hcaB, hcaC, hcaD, hcaE, hcaF, hcaR, hcaT, hdeA, hdeB, hdeD, hdhA, helD, hemA, hemB, hemC, hemD, hemE, hemF, hemG, hemH, hemK, hemL, hemM, hemX, hemY, hepA, het, hflB, hflC, hflK, hflX, hfq, hha, hipA, hipB, hisA, hisB, hisC, hisD, hisF, hisG, hisH, hisI, hisJ, hisM, hisP, hisQ, hisR, hisS, hipA, hlyE, hmp, hns, holA, holB, holC, holD, holE, hopB, hopC, hopD, hpt, hrpA, hrpB, hrsA, hscA, hscB, hsdM, hsdR, hsdS, hslC, hslD?, hslE-H, hslJ, hslK, hsIL-N, hslO-R, hslU, hslV, hslW, htgA, htpG, htpX, htrB, htrC, htrE, htrL, hupA, hupB, hyaA, hyaB, hyaC, hyaD, hyaE, hyaF, hybA, hybB, hybC, hybD, hybE, hybF, hybG, hycA, hycB, hycC, hycD, hycE, hycF, hycG, hycH, hycI, hydA, hydG, hydH, hydN, hyfA, hyfB, hyfC, hyfD, hyfE, hyfF, hyfG, hyfH, hyfI, hyfJ, hyfR, hypA, hypB, hypC, hypD, hypE, hypF, iadA, iap, ibpA, ibpB, icd, iclR, ihfA, ihfB, ileR, ileS, ileT, ileU, ileV, ileX, ileY, ilvA, ilvB, ilvC, ilvD, ilvE, ilvF, ilvG, ilvH, ilvI, ilvJ ilvM, ilvN, ilvR, ilvU, ilvY, imp, inaA, inaR?, infA, infB, infC, inm, insA(IS1), intA, isb(IS1), isfA, ispA, ispB, KanR, katE, katG, kba, kbl, kch, kdgK, kdgR, kdgT, kdpA, kdpB, kdpC, kdpD, kdpE, kdpF, kdsA, kdsB, kdtA, kdtB, kefB, kefC, kgtp, ksgA, ksgB, ksgC, ksgD, lacA, lacI, lacY, lacZ, lamB, lar, ldcC, ldhA, lepA, lepB, leuA, leuB, leuC, leuD, leuJ, leuO, leuP, leuQ, leuR, leuS, leuT, leuU, leuV, leuW, leuX, leuY, leuZ, lev, lexA, lgt, lhr, ligA, ligT, linB, lipA, lipB, lit, livF, livG, livH, livJ, livK, livM, lldD, lldP, lldR, lolA, lon, lpcA, lpcB, lpd, lplA, lpp, lpxA, lpxB, lpxC, lpxD, lpxK, lrb, lrhA, lrp, Irs lspA, lysA, lysC, lysP, lysQ, lysR, lysS, lysT, lysU, lysV,

lysW, lysX, lysY, lysZ, lytA, lytB, lyx, maa, mac, mae, mafA, mafB, malE, malF, malG, malI, malK, malM, malP, malQ, malS, malT, malX, malY, malZ, manA, manC, manX, manY, manZ, map, marA, marB, marR, mbrB, mcrA, mcrB, mcrC, mcrD, mdaB, mdh, mdoB, mdoG, mdoH, meb, melA, melB, melR, menA, menB, menC, menD, menE, menF, mepA, mesJ, metA, metB, metC, metD, metE, metF, metG, metH, metJ, metK, metL, metR, metT, metU, metV, metW, metY, metZ, mfd, mglA, mglB, mglC, mglR, mgsA, mgtA, mhpA, mhpB, mhpC, mhpD, mhpE, mhpF, mhpR, miaA, miaD, micF, minC, minD, minE, mioC, mltA, mltB, mltC, mltD, mmrA(rhlB?), mng, mntA, moaA, moaB, moaC, moaD, moaE, mobA, mobB, moc, modA, modB, modC, modE, modF, moeA, moeB, mog, molR, motA, motB, mpl, mppA, mprA, mraA--?, mraY, mrcA, mrcB, mrdA, mrdB, mreB, mreC, mreD, mrp, mrr, msbA, msbB, mscL, msrA, msyB, mtg, mtgA, mtlA, mtlD, mtlR, mtr, mttA, mttB, mttC, mukB, mukE, mukF, mul, murA, murB, murC, murD, murE, murF, murG, murH, murI, mutG(putative), mutH, mutL, mutM, mutS, mutT, mutY, nac, nadA, nadB, nadC, nadE, nagA, nagB, nagC, nagD, nagE, nalB, nalD, nanA, nanE, nanK, nanR, nanT, napA, napB, napC, napD, napF, napG, napH, narG, narH, narI, narJ, narK, narL, narP, narQ, narU, narV, narW, narX, narY, narZ, ndh, ndk, neaB, nei, nemA, nfi, nfnA, nfnB, nfo, nfrA, nfrB, nfrD, nfsA, nhaA, nhaB, nhaR, nikA, nikB, nikC, nikD, nikE, nirB, nirC, nirD, nlpA, nlpB, nlpC, nlpD, nmpC(qsr'), non, npr, nrdA, nrdB, nrdD, nrdE, nrdF, nrdG, nrfA, nrfB, nrfC, nrfD, nrfE, nrfF, nrfG, nth, ntpA, nuoA, nuoB, nuoC, nuoE, nuoF, nuoG, nuoH, nuoI, nuoJ, nuoK, nuoL, nuoM, nuoN, nupC, nupG, nusA, nusB, nusG, nuvA, nuvC, ogrK, ogt, ompA, ompC, ompF, ompG, ompR, ompT, ompX, oppA, oppB, oppC, oppD, oppE, oppF, opr, ops, oraA, ordL, orf-23(purB, reg)orfl95(nikA-reg), orn, osmB, osmC, osmE, osmY, otsA, otsB, oxyR, oxyS, pabA, pabB, pabC, pac, pal, panB, panC, panD, panF, parC, parE, pat, pbpG, pck, pcm, pcnB, pdhR, pdxA, pdxB, pdxH, pdxJ, pdxK, pdxL, pdxY, pepA, pepD, pepE, pepN, pepP, pepQ, pepT, pfkA, pfkB, pflA, pflB, pflC, pflD, pfs, pgi, pgk, pgl, pgm, pgpA, pgpB, pgsA, pheA, pheP, pheS, pheT, pheU, pheV, phnC, phnD, phnE, phnF, phnG, phnH, phnI, phnJ, phnK, phnL, phnM, phnN, phnO, phnP, phoA, phoB, phoE, phoH, phoP, phoQ, phoR, phoU,

phrB, phxB, pin, pioO, pit, pldA, pldB, plsB, plsC, plsX, pmbA, pncA, pncB, pnp, pntA, pntB, pnuC, poaR, polA, polB, popD, potA, potB, potC, potD, potE, potF, potG, potH, potI, poxA, poxB, ppa, ppc, pphA, pphB, ppiA, ppiB, ppiC, ppk, pppA, pps, ppx, pqiA, pqiB, pqqL, pqqM, prc, prfA, prfB, prfC, priA, priB, priC, prlC, prlZ, prmA, prmB, proA, proB, proC, proK, proL, proM, proP, proQ, proS, proT, proV, proW, proX, prpA, prpC, prpR, prr, prs, psd, psiF, pspA, pspB, pspC, pspE, pspF, pssA, pssR, pstA, pstB, pstC, pstS, psu, pta, pth, ptrA, ptrB, ptsG, ptsH, ptsI, ptsN"-", ptsP, purA, purB, purC, purD, purE, purF, purH, purK, purL, purM, purN, purP, purR, purT, purU, pus, putA, putP, pykA, pykF, pyrB, pyrC, pyrD, pyrE, pyrF, pyrG, pyrH, pyrI, qmeC, qmeD, qmeE, qor, queA, racC, racR, radA, radC, ranA, rarD, ras, rbfA, rbn, rbsA, rbsB, rbsC, rbsD, rbsK, rbsR, rcsA, rcsB, rcsC, rcsF, rdgA, rdgB, recA, recB, recC, recD, recE, recF, recG, recJ, recN, recO, recQ, recR, recT, relA, relB, relE, relF, relX, rep, rer, rfaB, rfaC, rfaD, rfaF, rfaG, rfaH, rfaI, rfaJ, rfaK, rfaL, rfaP, rfaQ, rfaS, rfaY, rfaZ, rfbA, rfbB, rfbC, rfbD, rfbX, rfc, rfe, rffA, rffC, rffD, rffE, rffG, rffH, rffM, rffT, rhaA, rhaB, rhaD, rhaR, rhaS, rhaT, rhlB, rhlE, rho, ribA, ribB, ribC, ribD, ribE, ribF, ridA, ridB, rimB, rimC, rimD, rimE, rimG, rimH, rimI, rimJ, rimK, rimL, rimM, rit, rlpA, rlpB, rluA, rluC, rluD, rmf, rna, rnb, rnc, rnd, rne, rnhA, rnhB, rnk, rnpA, rnpB, rnr, rnt, rob, rorB, rpe, rph, rpiA, rpiB, rpiR, rplA, rplB, rplC, rplD, rplE, rplF, rplI, rplJ, rplK, rplL, rplM, rplN, rplO, rplP, rplQ, rplR, rplS, rplT, rplU, rplV, rplW, rplX, rplY, rpmA, rpmB, rpmC, rpmD, rpmE, rpmF, rpmG, rpmH, rpmI, rpmJ, rpoA, rpoB, rpoC, rpoD, rpoE, rpoH, rpoN, rpoS, rpoZ, rpsA, rpsB, rpsC, rpsD, rpsE, rpsF, rpsG, rpsH, rpsI, rpsJ, rpsK, rpsL, rpsM, rpsN, rpsO, rpsP, rpsQ, rpsR, rpsS, rpsT, rpsU, rrfA, rrfB, rrfC, rrfD, rrfE, rrfF, rrfG, rrfH, rrlA, rrlB, rrlC, rrlD, rrlE, rrlG, rrlH, rrmA, rrsA, rrsB, rrsC, rrsD, rrsE, rrsG, rrsH, rsd, rseA, rseB, rseC, rspA, rspB, rssA, rssB, rsuA, rtcA, rtcB, rtcR, rtn, rus(qsr'), ruvA, ruvB, ruvC, sad, sanA, sapA, sapB, sapC, sapD, sapF, sbaA, sbcB, sbcC, sbcD, sbmA, sbmC(gyrI), sbp, sdaA, sdaB, sdaC, sdhA, sdhB, sdhC, sdhD, sdiA, sds, secA, secB, secD, secE, secF, secG, secY, selA, selB, selC, selD, semA, seqA, serA, serB, serC, serR serS, serT, serU, serV, serW, serX, sfa, sfcA, sfiC, sfsA, sfsB, shiA, sipC, sipD, sir, sixA, sloB, slp, slr, slt, slyD, slyX, smp,

smtA, sodA, sodB, sodC, sohA, sohB, solA, soxR, soxS, speA, speB, speC, speD, speE, speF, speG, spf, spoT, sppA, spr, srlA, srlB, srlD, srlE, srlR, srmB, srnA, ssaE, ssaG, ssaH, ssb, sseA, sseB, sspA, sspB, ssrA, ssrS, ssyA, ssyD stfZ, stkA, stkB, stkC, stkD, stpA, strC, strM, stsA, sucA, sucB, sucC, sucD, sufI, sugE, suhA, suhB, sulA, supQ, surA, surE, syd, tabC, tag, talA, talB, tanA, tanB, tap, tar, tas, tauA, tauB, tauC, tauD, tbpA, tdcA, tdcB, tdcC, tdcD, tdcE, tdcF, tdcG, tdcR, tdh, tdi tdk, tehA, tehB, tesA, tesB, tgt, thdA, thdC, thdD, thiB?, thiC, thiD, thiE, thiF, thiG, thiH, thiI, thiJ, thiK, thiL, thiM, thrA, thrB, thrC, thrS, thrT, thrU, thrV, thrW, thyA, tig, tktA, tktB, tldD, tlnA, tmk, tnaA, tnaB, tnaC, tnm, tol-orf1, tol-orf2, tolA, tolB, tolC, tolD, tolE, tolI, tolJ, tolM, tolQ, tolR, tonB, topA, topB, torA, torC, torD, torR, torS, torT, tpiA, tpr, tpx, treA, treB, treC, treF, treR, trg, trkA, trkD, trkG, trkH, trmA, trmB, trmC, trmD, trmE, trmF, trmH, trmU, trnA, trpA, trpB, trpC, trpD, trpE, trpR, trpS, trpT, truA, truB, trxA, trxB, trxC, tsaA, tsf, tsmA, tsr, tsx, ttdA, ttdB, ttk, tufA, tuffB, tus, tynA, tyrA, tyrB, tyrP, tyrR, tyrS, tyrT, tyrU, tyrV, ubiA, ubiB, ubiC, ubiD, ubiE, ubiF, ubiG, ubiH, ubiX, ucpA[], udk, udp, ugpA, ugpB, ugpC, ugpE, ugpQ, uhpA, uhpB, uhpC, uhpT, uidA, uidB, uidR, umuC, umuD, ung, upp, uppS, ups, uraA, usg-1, usbA, uspA, uup, uvh, uvrA, uvrB, uvrC, uvrD, uvs, uxaA, uxaB, uxaC, uxuA, uxuB, uxuR, valS, valT, valU, valV, valW, valX, valY, valZ, vsr, wrbA, xapA, xapB, xapR, xasA, xerC, xerD, xni, xseA, xseB, xthA, xylA, xylB, xylE, xylF, xylG, xylH, xylR, yccA, yhhP, yihG, yjaB, fl47, yjaD, yohF, yqiE, yrfE, zipA, zntA, znuA, znuB, znuC, zur, and zwf.

Non-limiting examples of mouse genes include: Ilr1, Ilr2, Gas10, Tnp1, Inhbb, Inha, Creb1, Mpmv34, Acrd , Acrg , Il110, Otf1, Rab11b-r, Abl1, ald, Amh-rs1, Bc12B , Cchlla3, Ccnb1-rs2, Gpcr16, Htr5b, Idd5, Igfbp2, Igfbp5, I18rb, Kras2-rs1, Mov7, Mpmv6, Mpmv16, Mpmv22, Mpmv25, Mpmv29, Mpmv42, Mtv7 , Mtv27, Mtv39, Oprk1, Otf3-rs1, Otf8, Otf11-rs1, Ptgs2, Ren1, Ren2 , Ril3, Sxv, Taz4-rs1, Tgfb2, Wnt6, Xmmv6 , Xmmv9, Xmmv36, Xmmv61, Xmmv74, Xmv21, Xmv32, Xmv41, I12ra, Ab1, Mpmv3, Rap1a-ps2, anx, Mpmv43, Ryr3, Ras12-4, Adra2b, Avp, Glvr1, Il1a, Il1b, Mpmv28, Oxt, Pcsk2, a, Xmv10, Tcf4 , Acra, Acra4, Ak1, Bdnf, bs, Cyct, Cyp24, Dbh, Fshb, Gcg, Gdf5 , Gnas, Gpcr8, Grin1, Hcs4,

Hior2, Hsp84-2, Idd12, Ilrn, Jund2, Kras3, Mc3r, Mpmv14, Mtv40, Mxi1-rs1, Otf3-rs2, Ptgs1, Ptpra, Rapsn, Src, Svp1, Svp3, Tcf3b, Wt1, Xmmv71, Xmv48, Ccna , Fgf2, Fth-rs1, Csfm , Mov10, Egf, Acrb2, Cap1, Crh, Fim3, Fps11, Glut2, Gpcr2, Gria2, Hsd3b-1, Hsd3b-2, Hsd3b-3, Hsd3b-4, Hsp86-ps2, Idd3, I12, I17, Mpvmv9, Mpmv20, Mtv4.8, Ngfb, Npra, Nras, Nras, Ntrk, Otf3-rs3, Otf3-rs4, Rap1a, Tshb, Xmmv22, Xmmv65, Mos, Ras12-7, Lyr, Ifa, Ifb, Jun, azh, db , Ipp , Mp1, Do1, Ak2, Ccnb1-rs4 , Cdc211, Cga, Fgr, Foc1, Fps12 , Gabrr1, Gabrr2, Gdf6, Glut1, Gnb1, Gpcr14, Grb2-ps, Grik3, Grik5, Hsp86-1ps4, Htr1da, Htr1db, Idd9, Ifa1, Ifa2, Ifa3, Ifa4, Ifa5, Ifa6, Ifa7, Ifa8, Ifa9, Ifa10, Lap18, Lmyc1, Mpmv19, Mpmv44, Mtv13 , Mtv14, Mtv17, Nppb, Otf6, Otf7, Ri12, Ski, Tnfr2, Wnt4, Xmmv8, Xmmv23, Xmmv62, Xmv1, Xmv2, Xmv8, Xmv9, Xmv14, Xmv44, Xpa, Tec, Fgf5, Nos1, Tcf1, Epo, Gnb2, Flt1, Flt3, Ache, Adra2c, Adrbk2, Afp, Alb1, Ccnb1-rs1, Clock, Cyp3 , Cyp3a11, Cyp3a13, Drd1b, Drd5, Fgfr3, Flk1, Gc, Gnrhr, Gpcr1, Hcs5, Hnf1, Htr5a, I15r, I16, Kit, Ltrm3, Mgsa, Mpmv7, Mpmv13, Mpmv23, Mtv32, Mtv41, Pdgfa, Pdgfra, Por, Txk, Xmmv3, Xmmv5, Xmmv52, Xmv17, Xmv28, Xmv34, Xmv38, Xmv45, Zp3, Trh, Raf1, Fth-rs2, Ntf3, Kras2, Pthlh, Mov1, Alox5, Braf2, Cftr, Egr4, Fpsl10, Fgf6, Gdf3, Ghrfr, Glut3, Grin2a, Hior3, Hoxa10, hop, Ica1, I15r, Int41, Itpr1, Krag, Mad, Met, Mi, Mtv8, Mtv23, Mtv29, Mtv33, Mtv34, Nkna, Npy, ob, Otf3-rs5, Tgfa, Tnfr1, Wnt2 , Wnt5B, Wnt7A, Xmmv27, Xmv24, Xmv61, Fosb, Ryr1, Ngfa, Ufo, Xrcc1, Abpa, Abpga, Gabra4, Gas2, Acra7, Ccnb1-rs7, Egfbp3, Xmv30, Zp2, Fes, Pcsk3, Calc, Ccnb1-rs10, Pth, Ad, Bc13, Cea, Cea2, Cea3, Cea4, Cea5, Cea6, Cebp, Dm9, Dm15, Drd4, Egfbp1, Egfbp2, Ercc2, Fgf3, Fgfr2, Gabra5, Gabrb3, Gtx, Hcs1, Igf1r, Igf2, I14r, Ins2, Int40, Lhb, Mpmv1, Mtv1, Mtv35, Ngfg, Ntf5, Otf2, 2, Pkcc, Ras14, Rras, Ryr, Svp2, Tcf3g, Tgfb1, tub, Xmmv31, Xmmv35, Xmmv73, Xmv33, Xmv53, Taz83, Adrb3, Junb, Jund1, Me1, Gpcr19-rs2, Agt, Cadp, Ccnb1-rs9, E, Fgfr1, Gas6, Gnb-rs1, Hcs2, Insr, Maf, Mov34, Mpmv21, Mpmv41, Mtv21, Mtnr1a, Plat, Ras15-2, Ras16, Sntb2, Xmmv29, Xmv12, Xmv26, Xmv62, Epor, Gpcr13, Otf11, Pthr, Acra3, Acra5, Acrb4, Camk1, Cdc25Mm, Crbp, Crbp2, Csk, Cyp11a, Cyp19, Drd2, Ets1, Fli1, Gnai2, Gnat1, Gpcr6, Gria4, Hgf1, Hior1, Hpx, Hsp86-1ps3, Hst2, Idd2,

Il1bc, Lag-rs1, Lap18-rs1, M11, Mpmv27, Penk, Pgr, Ras12-2, Tp11, Trf, Xmmv2, Xmmv67, Xmv15, Xmv16, Xmv25, Xmv60, Mgf, Amh, Braf, Cdc2a, Dmd1, Estr, Fps13 , Fps14 , Fps15 , Gli, Gpcr17, Grik2 , Ifgr, Igf1, Mpmv5, Mpmv12, Mpmv40, Myb, Oprm, Pg, Pmch, Ros1, Xmv31, Xmv51, Xmv54, Camk2b, Egfr, Int6, Lif, Mtv44, Ews, Csfgm, Flt4, I13, I14, I15, Irf1, Gria1, Glut4, Crhr, Csfg, Mov9, Xmv20, Acrb, Mpmv4, Mpmv15, Ngfr, Nos2, Rara, Taz4, Tcf2, Xmv42, Mtv3, Adra1, Crko, df, Erbb2, Gabra1, Gabra6, Gabrg2, Gh, Glra1, Grb2, Hnf1b, Hsp86-ps1, Idd4, Igfbp1, Igfbp3, I113, Int4, Mpmv2, Mpmv8, Mpmv18, Mtv45, nu, Pkca, Rab1, Rel, Shbg, Tcf7, Thra, Tnz1, Trp53, Wnt3, Wnt3A, Xmv4, Xmv5, Xmv47, Xmv49, Xmv63, Akt, Amh-rs4, Ccs1, Fps16, Fos, Gdf7, Hcs3, Hsp70-2, Hsp84-3, Hsp86-1, hyt, Ltrm1, Max, Mpmv11, Mpmv24, Mtv9, Mtv30, Pomc1, Tcf3a, Tda2, Tgfb3, Tpo, Tshr, Xmmv21, Xmmv25, Xmmv34, Xmmv50, Gli3, Xmv55, Ryr2, Inhba, Gas1, Pcsk1, Amh-rs2, Ccnb1-rs6, Ccnb1-rs13, Crhpb, Dat1, Drd1a, Fgfr4, Fps17, Fim1, Gpcr15, Gpcr18, Hbvi, Hilda, Htr1a, Idd11, I19, Ltrm4, Mak, mes, P11, P12, Pr1, Ra1, Rasa, Srd5a1, Tpbp, Xmv13, Xmv27, Rarb, Rbp3, Htr2, Rb1, Acra2, Camkg, Cch11a2, Ccnb1-rs5, Ccnb1-rs12, Gnrh, Mtv11, Nras-ps, Otf3-rs6, Plau, Ptprg, Trp53-ps, Wnt5A, Xmv19, Ghr, I17r, Lifr, Mlvi2, Prlr , Myc , Ril1, cog, Amh-rs7, I12rb, Pdgfb, Acr, CP2, Rarg, Sp1-1, Wnt1, Afr1, Atf4, Bzrp, Ccnb1-rs11, Cyp11b, I13rb1, I13rb2, Ins3, Itga, Mlvi1, Mlvi3, Mtv36, Pdgfec, Svp5, Tef, Trhr, Wnt7B, Xmmv55, Xmmv72, Xmv37, Tnp2, Ets2, Casr, Chuck-rs1, din, Drd3, Erg, G22p1, Gap43, Gas4, Grik1, Htr1f, Ifgt, İnt53, Ltrm2, Mpmv17, Mtv6, Mtvr1, Pit1, Xmv3, Xmv35, Xmv50, Igf2r, Mas, Tcd3, Glp1r, Idd1, Tla , Aeg1, Ccnb1-rs3, Cdc2b, Csi, Cyp21, Cyp21-ps1, Fps18 , Gna-rs1, Gpcr19-rs1, Grr1, Grr2, Hom1, Hsc70t, Hsp70, Hsp70-1, Hsp70-3, Hsp84-1, Hst1, Hst4, Hst5, Hst6, Hye, Int3, Itpr3, Lap18-rs2, Otf3, Ptprs, Rab11b, Ras12-1, Ras12-3, Ras13, Rrs, Rxrb, Tas, Tcd1, Tcd2, Tera1, Tla-rs, Tnfa, Tnfb, Tpx1, Tpx2, Xmmv15, Xmv36, Xmv57, Csfmr, Pdgfrb, Adrb2, Apc, Camk2a, Camk4, Dcc, Fgf1, Gna1, Gpcr7, Gr11, Grp, Hsp74, Mcc, Mtv2, Mtv38, Ptpn2, Tp12, Xmv22, Xmv23, Xmv29, Fth, Csfgmra, Mxi1, Adra2a, Adrb1, Adrbk1, Chuck, Cyp17, Gna14, Gnb-ps1, Hcs6, Htr7, Ide, Ins1, Lpc1, Pomc2, Seao, Tlx1, Xmmv42, Xmv18, Tcfe3, Araf, Avpr2, mdx, Ar,

Zfx, Otf9, Ccg1, Ccnb1-rs8, Fps19, Gabra3, Glra2, Glra4, Gria3, Grpr, Hsp74-ps1, Hst3, Htr1c, I12rg, Mov14, Mov15, Mtv28, Otf3-rs8, Sts, Sxa, Sxr, Xta, Tdy, Hya, Zfy1, Zfy2, Mov15, Mov24, Mtv31, Mtv42, Sdma, Spy, Sts, Sxa, Sxr, XmmvY, Xmv7, Xmv11, and Xmv40.

Non-limiting examples of Phaseolus vulgaris genes include: Acc, ace, Adk, Am, Amv-1, Amv-2, Ane, aph, Arc, Are, arg, Arl (Arc), asp, B, bc-u, bc-1.sup.1, bc-1.sup.2, bc-2.sup.1, bc-2.sup.2, bc-3 , Bcm, Beg, Bip, blu, Bpm, Bsm, By-1, By-2, C, C/c, c.sup.cr, C.sup.cir, C.sup.ma (M, R.sup.ma), C.sup.r, C.sup.res, C.sup.rho, C.sup.st, [C.sup.st R Acc] (Aeq), c.sup.u (inh, i.sub.e), [c.sup.u Prp.sup.i] (Prp, c.sup.ui, Nud), [c.sup.uprp.sup.st] (prp.sup.st), [C Prp] (Prp), c.sup.v, [C R] (R), [C r] (r), Ca, Cam, Cav, cc, ch1, c1, cm1, Co-1 (A), Co-2 (Are), Co-3 (Mexique 1), Co-3.sup.2, Co-4 (Mexique 2), Co-5 (Mexique 3), Co-6, Co-7, cr-1 cr-2, cry, cs, Ct, ctv-1 ctv-2, cyv (by-3), D (Can, Ins), Da, Db, def, dgs (g1, le), dia, Diap-1, Diap-2, diff, dis, D1-1 D1-2 (DL.sub.1 DL.sub.2), do, ds (te), dt-1.sup.a dt-2.sup.a, dt-1.sup.b dt-2.sup.b, dw-1 dw-2, Ea Eb, ers (restr), ers-2, Est-1, Est-2, exp, F, Fa, fast, Fb Fc, fa fb fc, Fcr, Fcr-2, fd, Fe-1 Fe-2, Fin (in), Fop-1, Fop-2, Fr, Fr-2, G (Flav, Ca, Och), Ga, gas, glb, Gpi-c1, Gr, Hb1 (L.sub.HB-1), Hbnc (SC.sub.HB-1), Hbp (PD.sub.HB-1), hmb, Hss, Hsw, Ht-1 Ht-2 (L-1 L-2), I, Ia Ib, ian-1 ian-2 (ia), Ibd, ico, Igr (Ih), ilo, ip, iter, iv, iw, J (Sh), Ke, L, la, Lan, Ld, Lds (Ds), Lec, Li (L), lo, Ir-1 lr-2, mar, Me, Mel (Me), Mel-2 (Me-2), mel-3 (me-3), Mf, mi, mia, Mic (Mip), miv, Mrf, Mrf.sup.2, mrf, ms-1, Mue, mu mutator, Nag, Nd-1 Nd-2 (D-1 D-2), nie, nnd (sym-1), nnd-2, No, nts (nod), Nudus, ol, P, p.sup.gri (Gri, v.sup.Pal), pa, pc, pg (pa.sub.1), Pha, Pmv, ppd (neu), Pr, prc (pc), Prx, punc, ram, Rbcs (rbcS), rf-1, rf-2, rf-3, rfi (i), Rfs (m), Rk, rk, rk.sup.d (lin), rn-1 rn-2 (r r), rnd, Ro, Sal, sb, sb.sup.ms, sb-2, sb-3, si1, Skdh, s1, Smv, St, Sur, sw-1 sw-2, T, t (z-1), Th-1 Th-2, Tm, To, Tor (T), Tr, tri, trv, Ts, tw, uni, Uni-2, uni.sup.nde, uni.sup.nie, Ur-1, Ur-2, Ur-2.sup.2, Ur-3 (Ur-3, Ur-4), Ur-3.sup.2, Ur-4, (Up-2, Ur-C), Ur-5, (B-190), Ur-6 (Ur.sub.a, Ur-G), Ur-7 (R.sub.B11), Ur-8 (Up-1), Ur-9 (Ur.sub.p), us, V (B1), v.sup.lae (Cor), v, var, vi (vir.sub.f), wb, Wmv, X.sup.su, y, and Z.

Non-limiting examples of Saccharomyces cerevisiae genes include: PRE3, PUP1, PUP3, PRE2, PRE10, PRE1, PRE8, SCL1, PUP2, PRE5, PRE7, PRE4, RPT2, RPT3, RPN3, RPN11, RPN12, RPT6, RPN1, RPN2, RPT1, RPT5, RPT4, SKI6, RRP4, DIS3, TSC10, RAT1, GND1, EXO70, ERG10, ACC1, RPP0, ACT1, ARP100, ARP3, PAN1, ARP2, ARP4, ARP9, SPE2, CYR1, ALA1, TPS1, TUB1, ABF1, DED81, NIP1, YHC1, SNU71, ATM1, MAK5, ROK1, DED1, SPB4, AUR1, PSE1, ALG1, TUB2, BPL1, MSL5, ERG24, ERG26, ERG25, CMD1, HCA4, SHE9, SHE10, CAK1, PIS1, CHO1, CDS1, ESR1, NUD1, CDC47, CDC13, CDC37, CDC1, CDC4, CDC20, CDC6, CDC46, CDC3, KAR1, BBP1, HRP1, CCT2, CCT3, HSP10, SMC1, SMC2, CHC1, CFT2, CLP1, COP1, SEC26, SEC27, RET2, SEC21, COF1, CCT4, CCT1, CCT6, SEC24, SEC7, PCF11, RNA15, RNA14, FIP1, YSH1, TFB4, TSM1, APC2, APC5, SEC31, TAF47, TAP42, MPP10, CDC53, CKS1, CDC28, KIN28, CNS1, ERG11, DBP10, DBP8, PRO3, DYS1, ALR1, TID3, DNA2, SSL2, RAD3, RFA3, RFA2, RFA1, RFC4, RFC5, RFC3, RFC2, RFC1, TOP2, RAP1, RPC25, PRI2, PRI1, POL1, POL12, HUS2, CDC2, POL2, DPB2, RPB10, RPA135, RPA190, RPA43, RPB8, RPO26, RPB5, RPC40, RPC19, SRB7, SRB4, RGR1, RPB11, SRB6, RPB2, RPB7, RPO21, RET1, RPO31, RPC31, RPC34, RPC53, RPC82, RPB12, RPB3, DPM1, DIP2, RNT1, CDC8, CDC14, DUT1, UBA2, UBA1, UBC9, CDC34, ENP1, ERD2, SSS1, SEC61, SEC63, SEC62, GNA1, GPI8, DAM1, DUO1, IRR1, PRP3, TIM9, HSH49, SUP35, EXM2, MEX67, ERG9, ERG20, FAS2, FAS1, NOP1, FAD1, AOS1, FBA1, NCB2, BRN1, TUB4, GDI1, GOG5, SRM1, CDC25, SPT16, YIF2, BET4, CDC43, MRS6, BET2, PRO1, GLN1, GLN4, GRS1, YIP1, FOL2, GPA1, CDC42, SAR1, YPT1, SEC4, GSP1, TEM1, RHO1, CDC24, RNA1, GUK1, VMA16, PMA1, HKR1, SIS1, MGE1, HSP60, HSF1, HAS1, MOT3, HTS1, ESA1, HSL7, HOM6, RIB7, SLY1, CSL4, PUR5, CSE1, IPP1, MDM1, USO1, SOF1, MAK11, LAS1, TEL2, DPB11, SGD1, FAL1, MTR3, MTR4, SPP2, SIK1, RRP7, POP4, RRP1, POP3, BFR2, CDC5, NRD1, MET30, MCM6, RRP46, SAS10, SCC2, ECO1, PRP43, BET3, BET5, STN1, NFS1, IDI1, SRP1, KAP95, CBF2, SKP1, CEP3, CTF13, ERG7, KRS1, PSA1, PMI40, ALG2, SSF1, MED7, RSC4, CDC54,

MCM2, AFG2, ERG12, MVD1, CDC48, MHP1, ERV1, SSC1, TIM44, TIM17, TIM23, TOM22, TOM40, MAS1, MCD1, MMC1, STU1, JAC1, ABD1, CEG1, PAB1, MTR2, SEC16, ROT1, INO1, MLC1, MYO2, GPI2, SPT14, NAT2, NMT1, TRM1, NCP1, NBP1, ACF2, SPP41, NUT2, LCP5, PRP19, NMD3, RFT1, NNF1, NDC1, CRM1, KAR2, NIP29, NAB2, NIC96, NUP145, NUP49, NUP57, NUP159, NSP1, NUP82, CDC39, NPL4, POP7, NTF2, MAK16, NPL3, NOP2, NOP4, NHP2, NOP10, GAR1, NBP35, WBP1, STT3, SWP1, OST2, OST1, ORC1, ORC6, ORC5, ORC4, ORC3, RRR1, SAT2, PWP2, PEX3, TOR2, PIK1, SEC14, STT4, MSS4, PCM1, GPM1, SEC53, ERG8, YPD1, PAP1, NAB3, RRN7, SEN1, CFT1, PRP11, PRP21, PRP39, PRP24, PRP9, SLU7, PRP28, PRP31, IFH1, PTA1, SUB2, FMI1, MAS2, ESS1, PFY1, POL30, POP1, PDI1, RAM2, CDC7, SMP3, CDC15, YTH1, QRI2, YAE1, SFI1, SEC1, BET1, SEC6, SEC13, SEC2, SEC8, CBF5, CDC19, YRB1, RHC18, DBF4, SDS22, MCM3, CEF1, ALG11, GAA1, MOB1, NIP7, TIP20, SEC5, SEC10, GPI10, RRP3, CDC45, DIB1, MIF2, HOP2, PBN1, NOP5, RPP1, POP5, POP8, POP6, ERO1, MPT1, DNA43, ESP1, SMC3, LST8, STS1, RPM2, RNR1, RNR2, RNR4, RPS20, RPL25, RPL3, RPL30, RPL32, RPL37A, RPL43A, RPL5, RPL10, RPS3, CET1, YRA1, SNM1, GLE1, DBP5, DRS1, DBP6, BRR2, RRN3, RRN6, RRN11, MED6, PRP16, RPR2, DIM1, RRP43, RRP42, RRP45, SEC20, BOS1, CDC12, GLC7, PKC1, IPL1, SGV1, NRK1, RAD53, LCB2, LCB1, MPS1, SES1, SPC3, SEC11, RIO1, ARP7, NEO1, YJU2, POB3, ARH1, IQG1, HRT1, HYM1, MAK21, FUN20, FUN9, NBN1, STB5, YIF1, SMX4, YKT6, SFT1, SMD1, PRP6, LSM2, NUF1, SPC97, SPC42, SPC98, CDC31, SPC19, SPC25, SPC34, SPC24, NUF2, PRP40, MCD4, ERG1, SMC4, CSE4, KRR1, SME1, TRA1, RLP7, SCH9, SMD3, SNP2, SSF2, SPC72, CDC27, CDC23, CDC16, APC1, APC11, APC4, ARC19, RPN6, RPN5, RSC6, RSC8, STH1, SFH1, TIM12, TIM22, TIM10, SQT1, SLS1, JSN1, STU2, SCD5, SSU72, ASM4, SED5, UFE1, SYF1, SYF2, CCT5, TBF1, TOA2, TOA1, SUA7, TAF90, TAF61, TAF25, TAF60, TAF17, TAF145, TAF19, TAF40, TAF67, TFA2, TFA1, FCP1, TFG1, TFG2, TFB1, CCL1, SSL1, TFB3, TFB2, PZF1, BRF1, TFC5, TFC4, TFC3, TFC7, TFC6, TFC1, SPT15, THI80, THS1, SPT6, SPT5, ROX3, REB1,

MCM1, MED4, MOT1, MED8, EFB1, YEF3, SUI1, CDC95, TIF11, SUI3, GCD11, SUI2, GCD6, GCD7, GCD2, GCD1, RPG1, GCD10, PRT1, TIF34, CDC33, TIF5, SUP45, GCD14, TIM54, SEC17, TPT1, TRL1, CCA1, SEN54, SEN2, SEN15, SEN34, WRS1, SLN1, TYS1, SNU56, PRP42, CUS1, PRP4, PRP8, SNU114, USS1, UFD1, SMT3, RSP5, QRI1, ALG7, UGP1, VTI1, VAS1, SEC18, CTR86, and ZPR1.

2. Viruses

The microorganisms provided herein include viruses. Such viruses typically have one or more of the microorganism characteristics provided herein. For example, viruses provided herein can have attenuated pathogenicity, reduced toxicity, preferential accumulation in immunoprivileged cells and tissues, such as tumor, ability to activate an immune response against tumor cells, immunogenic, replication competent, and are able to express exogenous proteins, and combinations thereof. In some embodiments, the viruses have an ability to activate an immune response against tumor cells without aggressively killing the tumor cells.

The viruses provided herein can be cytoplasmic viruses, such as poxviruses, or can be nuclear viruses such as adenoviruses. The viruses provided herein can have as part of their life cycle lysis of the host cell's plasma membrane. Alternatively, the viruses provided herein can have as part of their life cycle exit of the host cell by non-lytic pathways such as budding or exocytosis. The viruses provided herein can cause a host organism to develop an immune response to virus-infected tumor cells as a result of lysis or apoptosis induced as part of the viral life cycle. The viruses provided herein also can be genetically engineered to cause a host organism to develop an immune response to virus-infected tumor cells as a result of lysis or apoptosis, regardless of whether or not lysis or apoptosis is induced as part of the viral life cycle. In some embodiments, the viruses provided herein can cause the host organism to mount an immune response against tumor cells without lysing or causing cell death of the tumor cells.

One skilled in the art can select from any of a variety of viruses, according to a variety of factors, including, but not limited to, the intended use of the virus (*e.g.*,

exogenous protein production, antibody production or tumor therapy), the host organism, and the type of tumor.

a. Cytoplasmic viruses

The viruses provided herein can be cytoplasmic viruses, where the life cycle of the virus does not require entry of viral nucleic acid molecules in to the nucleus of the host cell. A variety of cytoplasmic viruses are known, including, but not limited to, pox viruses, African swine flu family viruses, and various RNA viruses such as picorna viruses, calici viruses, toga viruses, corona viruses and rhabdo viruses. In some embodiments, viral nucleic acid molecules do not enter the host cell nucleus throughout the viral life cycle. In other embodiments, the viral life cycle can be performed without use of host cell nuclear proteins. In other embodiments, the virulence or pathogenicity of the virus can be modulated by modulating the activity of one or more viral proteins involved in viral replication.

i. Poxviruses

In one embodiment, the virus provided herein is selected from the pox virus family. Pox viruses include Chordopoxvirinae such as orthopoxvirus, parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, molluscipoxvirus and yatapoxvirus, as well as Entomopoxvirinae such as entomopoxvirus A, entomopoxvirus B, and entomopoxvirus A. Chordopoxvirinae are vertebrate poxviruses and have similar antigenicities, morphologies and host ranges; thus, any of a variety of such poxviruses can be used herein. One skilled in the art can select a particular genera or individual chordopoxvirinae according to the known properties of the genera or individual virus, and according to the selected characteristics of the virus (*e.g.*, pathogenicity, ability to elicit and immune response, preferential tumor localization), the intended use of the virus, the tumor type and the host organism. Exemplary chordopoxvirinae genera are orthopoxvirus and avipoxvirus.

Avipoxviruses are known to infect a variety of different birds and have been administered to humans. Exemplary avipoxviruses include canarypox, fowlpox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox, penguinpox, sparrowpox, starlingpox, and turkeypox viruses.

Orthopoxviruses are known to infect a variety of different mammals including rodents, domesticated animals, primates and humans. Several orthopoxviruses have a broad host range, while others have narrower host range. Exemplary orthopoxviruses include buffalopox, camelpox, cowpox, ectromelia monkeypox, raccoon pox, skunk pox, tatera pox, uasin gishu, vaccinia, variola and volepox viruses. In some embodiments, the orthopoxvirus selected can be an orthopoxvirus known to infect humans, such as cowpox, monkeypox, vaccinia or variola virus. Optionally, the orthopoxvirus known to infect humans can be selected from the group of orthopoxviruses with a broad host range, such as cowpox, monkeypox, or vaccinia virus.

a. Vaccinia Virus

One exemplary orthopoxvirus is vaccinia virus. A variety of vaccinia virus strains are available, including Western Reserve (WR), Copenhagen, Tashkent, Tian Tan, Lister, Wyeth, IHD-J, and IHD-W, Brighton, Ankara, MVA, Dairen I, L-IPV, LC16M8, LC16MO, LIVP,WR 65-16, Connaught, New York City Board of Health. Exemplary vaccinia viruses are Lister or LIVP vaccinia viruses. Any known vaccinia virus, or modifications thereof that correspond to those provided herein or known to those of skill in the art to reduce toxicity of a vaccinia virus. Generally, however, the mutation will be a multiple mutant and the virus will be further selected to reduce toxicity.

The linear dsDNA viral genome of vaccinia virus is approximately 200 kb in size, encoding a total of approximately 200 potential genes. Viral gene expression can be divided into three stages. In the early stage, gene expression is mainly for viral replication, and for defense against the host's immune system. In the intermediate stage, genes not available for expression in the early stage can be expressed, including late stage transactivators. In the late stage, active transcription is mainly for viral structural components for building mature viruses.

Vaccinia virus possesses a variety of features for use in cancer gene therapy and vaccination. It has a broad host and cell type range. Vaccinia is a cytoplasmic virus, thus, it does not insert its genome into the host genome during its life cycle.

Unlike many other viruses that require the host's transcription machinery, vaccinia virus can support its own gene expression in the host cell cytoplasm using enzymes encoded in the viral genome. The vaccinia virus genome has a large carrying capacity for foreign genes, where up to 25 kb of exogenous DNA fragments (approximately 12% of the vaccinia genome size) can be inserted. The genomes of several of the vaccinia strains have been completely sequenced, and many essential and nonessential genes identified. Due to high sequence homology among different strains, genomic information from one vaccinia strain can be used for designing and generating modified viruses in other strains. Finally, the techniques for production of modified vaccinia strains by genetic engineering are well established (Moss, Curr. Opin. Genet. Dev. 3 (1993), 86-90; Broder and Earl, Mol. Biotechnol. 13 (1999), 223-245; Timiryasova *et al.*, Biotechniques 31 (2001), 534-540).

Historically, vaccinia virus was used to immunize against smallpox infection. More recently, modified vaccinia viruses are being developed as vaccines to combat a variety of diseases. Attenuated vaccinia virus can trigger a cell-mediated immune response. Strategies such as prime/boost vaccination, vaccination with nonreplicating vaccinia virus or a combination of these strategies, have shown promising results for the development of safe and effective vaccination protocols. Mutant vaccinia viruses from previous studies exhibit a variety of shortcomings, including a lack of efficient delivery of the viral vehicle to the desired tissue only (e.g., specific accumulation in a tumors), a lack of safety because of possible serious complications (*e.g.*, in young children, eczema vaccinatum and encephalitis, and in adults disseminated or progressive vaccinia may result if the individual is severely immunodeficient).

b. Modified Vaccinia Viruses

Provided herein are vaccinia viruses with insertions, mutations or deletions, as described more generally elsewhere herein. The vaccinia viruses are modified or selected to have low toxicity and to accumulate in the target tissue. Exemplary of such viruses are those from the LIVP strain.

Exemplary insertions, mutations or deletions are those that result in an attenuated vaccinia virus relative to the wild type strain. For example, vaccinia virus insertions, mutations or deletions can decrease pathogenicity of the vaccinia virus, for example, by reducing the toxicity, reducing the infectivity, reducing the ability to replicate, or reducing the number of non-tumor organs or tissues to which the vaccinia virus can accumulate. Other exemplary insertions, mutations or deletions include, but are not limited to, those that increase antigenicity of the microorganism, those that permit detection or imaging, those that increase toxicity of the microorganism (optionally, controlled by an inducible promoter). For example, modifications can be made in genes that are involved in nucleotide metabolism, host interactions and virus formation. Any of a variety of insertions, mutations or deletions of the vaccinia virus known in the art can be used herein, including insertions, mutations or deletions of: the thymidine kinase (TK) gene, the hemagglutinin (HA) gene, the VGF gene (as taught in U.S. Pat. Pub. No. 20030031681); a hemorrhagic region or an A type inclusion body region (as taught in U.S. Pat. No. 6,596,279); Hind III F, F13L, or Hind III M (as taught in U.S. Pat. No. 6,548,068); A33R, A34R, A36R or B5R genes (see, *e.g.*, Katz *et al.*, J. Virology 77:12266-12275 (2003)); SalF7L (see, *e.g.*, Moore *et al.*, EMBO J. 1992 11:1973-1980); N1L (see, *e.g.*, Kotwal *et al.*, Virology 1989 171:579-587); M1 lambda (see, *e.g.*, Child *et al.*, Virology. 1990 174:625-629); HR, HindIII-MK, HindIII-MKF, HindIII-CNM, RR, or BamF (see, *e.g.*, Lee *et al.*, J Virol. 1992 66:2617-2630); or C21L (see, *e.g.*, Isaacs *et al.*, Proc Natl Acad Sci U S A. 1992 89:628-632).

c. The F3 Gene

In addition to the mutations known in the art, the vaccinia viruses provided herein can have an insertion, mutation or deletion of the F3 gene (SEQ ID No: 1; an exemplary F3 gene is provided in GenBank Accession No. M57977, which contains the nucleotide and predicted amino acid sequences for LIVP strain F3; see also Mikryukov *et al.*, Biotekhnologiya 4:442-449 (1988)). For example, the F3 gene has been modified at the unique single NotI restriction site located within the F3 gene at position 35 or at position 1475 inside of the HindIII-F fragment of

vaccinia virus DNA strain LIVP (Mikryukov *et al.*, Biotekhnologiy 4 (1988), 442-449) by insertion of a foreign DNA sequence into the NotI digested virus DNA. As provided herein, an insertion of a nucleic acid molecule containing lacZ or luciferase/GFP into the NotI site of the F3 gene of the LIVP strain (nucleotides 1473-1480 in M57977, or nucleotides 33-40 of SEQ ID NO: 1) can result in decreased accumulation of vaccinia viruses in non-tumorous organs of nude mice, including brain and heart, relative to wild type vaccinia virus. Thus for use in the methods provided herein, vaccinia viruses can contain an insertion, mutation or deletion of the F3 gene or a mutation of a corresponding locus.. For example, as provided herein, F3-interrupted modified LIVP vaccinia virus can selectively replicate in tumor cells in vivo. Therefore, modified vaccinia viruses (*e.g.*, modified strain LIVP) with the interrupted F3 gene can be used in the methods provided herein, such as methods of tumor-directed gene therapy and for detection of tumors and metastases.

Thus, provided herein are vaccinia viruses having a modification of the F3 gene. For example, the vaccinia viruses provided herein can contain an insertion of foreign DNA into the F3 gene. An exemplary insertion of foreign DNA is an insertion at a site equivalent to the NotI site of the F3 gene in vaccinia strain LIVP, or at position 35 of SEQ ID No:1. An F3-modified vaccinia virus provided herein can colonize in tumors specifically, and therefore, can be used for tumor-specific therapeutic gene delivery. A GenBank data analysis with BLAST (Basic Local Alignment Search Tool) on nucleotide sequences of different strains of vaccinia virus was performed. Based on this analysis, it was found that in vaccinia virus strain Copenhagen (Goebel *et al.*, Virology 179 (1990), 247-266) the NotI restriction site is located between two open reading frames (ORF) encoding F14L and F15L genes. Therefore, insertion of foreign genes into NotI site of the VV genome strain Copenhagen will not interrupt any vital genes. In VV strain LIVP, the NotI restriction site is located in the ORF encoding the F3 gene with unknown function (Mikryukov *et al.*, Biotekhnologiya 4 (1988), 442-449). Thus, the insertion of foreign genes into the NotI site of the F3 gene interrupted the F3 gene. The ability

to modify the F3 gene suggests that it may have a nonessential role for virus replication. Although the F3 gene is likely nonessential for virus replication, the results of the animal experiments suggest that interruption of the F3 gene is correlated with decreased viral virulence, the inability to replicate in brain or ovary, and the ability to replicate preferentially in tumor tissue.

The F3 gene is conserved in a variety of different vaccinia virus strains, including WR (nucleotides 42238-42387 of GenBank Accession No. AY243312.1, Ankara (nucleotides 37155-37304 of GenBank Accession No. U94848.1), Tian Tan (nucleotides 41808-41954 of GenBank Accession No. AF095689), Acambis 3000 (nucleotides 31365-31514 of GenBank Accession No. AY603355.1) and Copenhagen (nucleotides 45368-45517 of GenBank Accession No. M35027.1) strains. The F3 gene also is conserved in the larger family of poxviruses, particularly among orthopoxviruses such as cowpox (nucleotides 58498-58647 of GenBank Accession No. X94355.2), rabbitpox (nucleotides 46969-47118 of GenBank Accession No. AY484669.1), camelpox (nucleotides 43331-43480 of GenBank Accession No. AY009089.1), ectromelia (nucleotides 51008-51157 of GenBank Accession No. AF012825.2), monkeypox (nucleotides 42515-42660 of GenBank Accession No. AF380138.1), and variola viruses (nucleotides 33100-33249 of GenBank Accession No. X69198.1). Accordingly, also provided are modifications of the equivalent of the F3 gene in poxviruses, such as orthopoxviruses including a variety of vaccinia virus strains. One skilled in the art can identify the location of the equivalent F3 gene in a variety of poxviruses, orthopoxviruses and vaccinia viruses. For example, an equivalent of the F3 gene in poxviruses, orthopoxviruses and vaccinia viruses can include a gene that contains at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the nucleotide sequence of the F3 gene in SEQ ID No:1. In another example, an equivalent of the F3 gene in poxviruses, orthopoxviruses and vaccinia viruses can include a gene that contains at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity with the amino

acid sequence of F3 in SEQ ID No:2. In another example, the equivalent to the F3 gene in LIVP can be determined by its structural location in the viral genome: the F3 gene is located on the HindIII-F fragment of vaccinia virus between open reading frames F14L and F15L as defined by Goebel *et al.*, Virology (1990) 179:247-266, and in the opposite orientation of ORFs F14L and F15L; one skilled in the art can readily identify the gene located in the structurally equivalent region in a large variety of related viruses, such as a large variety of pox viruses.

Comparative protein sequence analysis revealed some insight into protein function. The closest match with the protein encoded by the F3 gene (strain LIVP) is a prolyl 4-hydroxylase alpha subunit precursor (4-PH alpha) from the nematode Caenorhabditis elegans (Veijola *et al.*, J. Biol. Chem. 269 (1994), 26746-26753). This alpha subunit forms an active alpha-beta dimer with the human protein disulfide isomerase beta subunit. Prolyl 4-hydroxylase (EC 1.14.11.2) catalyzes the formation of 4-hydroxyproline in collagen. The vertebrate enzyme is an alpha 2-beta 2 tetramer, the beta subunit of which is identical to the protein disulfide-isomerase (PDI). The importance of this protein for vaccinia viral replication is unknown, but a deficiency of this protein can result in retargeting vaccinia virus to tumor tissue.

d. Multiple Modifications

The vaccinia viruses provided herein also can contain two or more insertions, mutations or deletions. Thus, included are vaccinia viruses containing two or more insertions, mutations or deletions of the loci provided herein or other loci known in the art. In one embodiment, a vaccinia virus contains an insertion, mutation or deletion in the F3 gene, and one or more additional insertions, mutations or deletions. In one embodiment of the modified vaccinia virus, at least the F3 gene has been modified by insertion of a foreign nucleotide sequence. Modifications such as modification of the F3 gene will typically result in at least partial inactivation of the gene or gene product. In one example, the F3 gene and the TK gene have been modified by insertion of a foreign nucleotide sequence. In another example, the F3 gene and the HA gene have been modified by insertion of a foreign nucleotide

sequence. In another example, the F3 gene and both the TK and HA genes have been modified by insertion of a foreign nucleotide sequence. In another example, the HA gene and the TK gene have been modified by insertion of a foreign nucleotide sequence. Accordingly, the present compositions and methods include a modified vaccinia virus wherein two or more of (a) the F3 gene, (b) the TK gene, and (c) the HA gene have been modified. In one embodiment, at least two of the F3 gene, TK gene and HA gene have been inactivated, for example by insertion, deletion and/or replacement of nucleotide(s) within the coding region, or regulatory sequences of two or more of these genes have been inactivated by insertion, deletion or mutation.

e. The Lister Strain

In another embodiment, the viruses and methods provided herein can be based on modifications to the Lister strain of vaccinia virus. Lister (also referred to as Elstree) vaccinia virus is available from any of a variety of sources. For example, the Elstree vaccinia virus is available at the ATCC under Accession Number VR-1549. The Lister vaccinia strain has high transduction efficiency in tumor cells with high levels of gene expression.

In one embodiment, the Lister strain can be an attenuated Lister strain, such as the LIVP (Lister virus from the Institute of Viral Preparations, Moscow, Russia) strain, which was produced by further attenuation of the Lister strain. The LIVP strain was used for vaccination throughout the world, particularly in India and Russia, and is widely available.

The LIVP strain has a reduced pathogenicity while maintaining a high transduction efficiency. For example, as provided herein, F3-interrupted modified LIVP vaccinia virus can selectively replicate in tumor cells in vivo. In one embodiment, provided herein are modified LIVP viruses, including viruses having a modified TK gene, viruses having a modified HA gene, viruses having a modified F3 gene, and viruses having two or more of: modified HA gene, modified TK gene, and modified F3 gene.

ii. Other cytoplasmic viruses

Also provided herein are cytoplasmic viruses that are not poxviruses. Cytoplasmic viruses can replicate without introducing viral nucleic acid molecules into the nucleus of the host cell. A variety of such cytoplasmic viruses are known in the art, and include African swine flu family viruses and various RNA viruses such as arenaviruses, picornaviruses, caliciviruses, togaviruses, coronaviruses, paramyxoviruses, flaviviruses, reoviruses, and rhaboviruses. Exemplary togaviruses include Sindbis viruses. Exemplary arenaviruses include lymphocytic choriomeningitis virus. Exemplary rhaboviruses include vesicular stomatitis viruses. Exemplary paramyxo viruses include Newcastle Disease viruses and measles viruses. Exemplary picornaviruses include polio viruses, bovine enteroviruses and rhinoviruses. Exemplary flaviviruses include Yellow fever virus; attenuated Yellow fever viruses are known in the art, as exemplified in Barrett *et al.*, Biologicals 25:17-25 (1997), and McAllister *et al.*, J. Virol. 74:9197-9205 (2000).

Also provided herein are modifications of the viruses provided above to enhance one or more characteristics relative to the wild type virus. Such characteristics can include, but are not limited to, attenuated pathogenicity, reduced toxicity, preferential accumulation in tumor, increased ability to activate an immune response against tumor cells, increased immunogenicity, increased or decreased replication competence, and are able to express exogenous proteins, and combinations thereof. In some embodiments, the modified viruses have an ability to activate an immune response against tumor cells without aggressively killing the tumor cells. In other embodiments, the viruses can be modified to express one or more detectable genes, including genes that can be used for imaging. In other embodiments, the viruses can be modified to express one or more genes for harvesting the gene products and/or for harvesting antibodies against the gene products.

b. Adenovirus, Herpes, Retroviruses

Further provided herein are viruses that include in their life cycle entry of a nucleic acid molecule into the nucleus of the host cell. A variety of such viruses are

known in the art, and include herpesviruses, papovaviruses, retroviruses, adenoviruses, parvoviruses and orthomyxoviruses. Exemplary herpesviruses include herpes simplex type 1 viruses, cytomegaloviruses, and Epstein-Barr viruses. Exemplary papoviruses include human papillomaviruses and SV40 viruses. Exemplary retroviruses include lentiviruses. Exemplary orthomyxoviruses include influenza viruses. Exemplary parvoviruses include adeno associated viruses.

Also provided herein are modifications of the viruses provided above to enhance one or more characteristics relative to the wild type virus. Such characteristics can include, but are not limited to, attenuated pathogenicity, reduced toxicity, preferential accumulation in tumor, increased ability to activate an immune response against tumor cells, increased immunogenicity, increased or decreased replication competence, and are able to express exogenous proteins, and combinations thereof. In some embodiments, the modified viruses have an ability to activate an immune response against tumor cells without aggressively killing the tumor cells. In other embodiments, the viruses can be modified to express one or more detectable genes, including genes that can be used for imaging. In other embodiments, the viruses can be modified to express one or more genes for harvesting the gene products and/or for harvesting antibodies against the gene products.

3. Bacteria

Bacteria can also be used in the methods provided herein. Any of a variety of bacteria possessing the desired characteristics can be used. In one embodiment, aerobic bacteria can be used. In another embodiment, anaerobic bacteria can be used. In another embodiment, extracellular bacteria can be used. In another embodiment, intracellular bacteria can be used.

In some embodiments, the bacteria provided herein can be extracellular bacteria. A variety of extracellular bacteria are known in the art and include vibrio, lactobacillus, streptococcus, escherichia. Exemplary bacteria include Vibrio cholerae, Streptococcus pyogenes, and Escherichia coli. In other embodiments, the bacteria provided herein can be intracellular bacteria. A variety of intracellular

bacteria are known in the art and include listeria, salmonella, clostridium, and bifodobacterium. Exemplary intracellular bacteria include Listeria monocytogenes, Salmonella typhimurium, Clostridium histolyticus, Clostridium butyricum, Bifodobacterium longum, and Bifodobacterium adolescentis. Additional bacteria include plant bacteria such as Clavibacter michiganensis subsp. michiganensis, Agrobacterium tumefaciens, Erwinia herbicola, Azorhizobium caulinodans, Xanthomonas campestris pv. vesicatoria, and Xanthomonas campestris pv. campestris.

A further example of a bacteria provided herein are magnetic bacteria. Such bacteria allow tumor detection through the accumulation of iron-based contrast agents. Magnetic bacteria can be isolated from fresh and marine sediments. Magnetic bacteria can produce magnetic particles (Fe304) (Blakemore, Annu. Rev. Microbiol. 36 (1982), 217-238). To do so, the magnetic bacteria have efficient iron uptake systems, which allow them to utilize both insoluble and soluble forms of iron. Magnetospirillum magnetic AMB-1 is an example of such magnetic bacteria that has been isolated and cultured for magnetic particle production (Yang *et al.*, Enzyme Microb. Technol. 29 (2001), 13-19). As provided herein, these magnetic bacteria (naturally occurring or genetically modified), when injected intravenously, can selectively accumulate in tumor. Accordingly, these bacteria can be used for accumulating iron-based contrast agents in the tumors, which in turn allows tumor detection by MRI. Similarly, other naturally isolated metal accumulating strains of bacteria can be used for tumor targeting, absorption of metals from contrast agents, and tumor imaging.

Also provided herein are modifications of bacteria to enhance one or more characteristics relative to the wild type bacteria. Such characteristics can include, but are not limited to, attenuated pathogenicity, reduced toxicity, preferential accumulation in tumor, increased ability to activate an immune response against tumor cells, increased immunogenicity, increased or decreased replication competence, and are able to express exogenous proteins, and combinations thereof. In some embodiments, the modified bacteria have an ability to activate an immune

response against tumor cells without aggressively killing the tumor cells. In other embodiments, the bacteria can be modified to express one or more detectable genes, including genes that can be used for imaging. In other embodiments, the bacteria can be modified to express one or more genes for harvesting the gene products and/or for harvesting antibodies against the gene products.

a. Aerobic bacteria

Previous studies have postulated that anaerobic bacteria are preferred for administration to tumors (Lemmon *et al.*, 1997 Gene Therapy 4:791-796). As provided herein, it has been determined that aerobic bacteria can survive and grow in tumors. Accordingly, a bacteria used in the methods provided herein can include a bacteria that can survive and grow in an oxygenated environment. In some embodiments, the bacteria must be in an oxygenated environment in order to survive and grow. A variety of aerobic bacteria are known in the art, including lactobacilli, salmonella, streptococci, staphylococci, vibrio, listeria and escherichia. Exemplary bacteria include Vibrio cholerae, Listeria monocytogenes, Salmonella typhimurium, Streptococcus pyogenes, Escherichia coli, Lactobacillus bulgaricus, Lactobacillus casei, Lacto bacillus acidophilus, Lactobacillus brevis, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus sporogenes, Lactobacillus lactis, Lactobacillus fermentum, Streptococcus thermophilus, Bacillus subtilis, Bacillus megaterium, Bacillus polymyxa, Myobacterium smegmatis, Mycobacterium vaccae, Mycobacterium microti, Mycobacterium habana, Enterococcus faecalis, Pseudomonas fluorescens, and Pseudomonas putida.

b. Anaerobic bacteria

A bacteria used in the methods provided herein can include a bacteria that does not require oxygen to survive and grow. In some embodiments, the bacteria must be in an oxygen-free environment in order to survive and grow. A variety of aerobic bacteria are known in the art, including clostridium, bifodobacterium. Exemplary bacteria include Clostridium histolyticus, Clostridium butyricum, Clostridium novyi, Clostridium sordellii, Clostridium absonum, Clostridium

bifermentans, Clostridium difficile, Clostridium histolyticum, Clostridium perfringens, Clostridium beijerinckii, Clostridium sporogenes, Staphylococcus aureus, Staphylococcus epidermidis, Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium laterosporus, Bifidobacterium animalis, Actinomyces israelii, Eubacterium lentum, Peptostreptococcus anaerobis, Peptococcus prevotti, and Acidaminococcus fermentans.

4. Eukaryotic cells

Also encompassed within the microorganisms provided herein and the methods of making and using such microorganisms are eukaryotic cells, including cells from multicellular eukaryotes, including mammals such as primates, where exemplary cells are human cells. Typically the cells are isolated cells. For example, eukaryotic cells can be tumor cells, including mammalian tumor cells such as primate tumor cells, where exemplary primate tumor cells are human tumor cells such as human breast cancer cells. In another example, eukaryotic cells can include fibrosarcoma cells such as human fibrosarcoma cells. Exemplary human fibrosarcoma cells include HT1080 (ATCC Accession Nos. CCL-121, CRL-12011 or CRL-12012). In another example, eukaryotic cells can include stem cells, including mammalian stem cells such as primate stem cells, where exemplary primate stem cells are human stem cells.

Also provided herein are modifications of eukaryotic cells to enhance one or more characteristics relative to the wild type cells. Such characteristics can include, but are not limited to, attenuated pathogenicity, reduced toxicity, preferential accumulation in tumor, increased ability to activate an immune response against tumor cells, increased immunogenicity, increased or decreased replication competence, and are able to express exogenous proteins, and combinations thereof. In some embodiments, the modified eukaryotic cells have an ability to activate an immune response against tumor cells without aggressively killing the tumor cells. In other embodiments, the eukaryotic cells can be modified to express one or more detectable genes, including genes that can be used for imaging. In other

embodiments, the eukaryotic cells can be modified to express one or more genes for harvesting the gene products and/or for harvesting antibodies against the gene products.

C. Methods for Making a Modified Microorganism

The microorganisms provided herein can be formed by standard methodologies well known in the art for modifying microorganisms such as viruses, bacteria and eukaryotic cells. Briefly, the methods include introducing into microorganisms one or more genetic modification, followed by screening the microorganisms for properties reflective of the modification or for other desired properties.

1. Genetic Modifications

Standard techniques in molecular biology can be used to generate the modified microorganisms provided herein. Such techniques include various nucleic acid manipulation techniques, nucleic acid transfer protocols, nucleic acid amplification protocols, and other molecular biology techniques known in the art. For example, point mutations can be introduced into a gene of interest through the use of oligonucleotide mediated site-directed mutagenesis. Alternatively, homologous recombination can be used to introduce a mutation or exogenous sequence into a target sequence of interest. Nucleic acid transfer protocols include calcium chloride tranformation/transfection, electroporation, liposome mediated nucleic acid transfer, N-[1-(2,3-Dioloyloxy)propyl]-N,N,N-trimethylammonium methylsulfate meditated transformation, and others. In an alternative mutagenesis protocol, point mutations in a particular gene can also be selected for using a positive selection pressure. See, *e.g.*, Current Techniques in Molecular Biology, (Ed. Ausubel, *et al.*). Nucleic acid amplification protocols include but are not limited to the polymerase chain reaction (PCR). Use of nucleic acid tools such as plasmids, vectors, promoters and other regulating sequences, are well known in the art for a large variety of viruses and cellular organisms. Further a large variety of nucleic acid tools are available from many different sources including ATCC, and various commercial sources. One skilled in the art will be readily able to select the

appropriate tools and methods for genetic modifications of any particular virus or cellular organism according to the knowledge in the art and design choice.

Any of a variety of modifications can be readily accomplished using standard molecular biological methods known in the art. The modifications will typically be one or more truncations, deletions, mutations or insertions of the microorganismal genome. In one embodiment, the modification can be specifically directed to a particular sequence. The modifications can be directed to any of a variety of regions of the microorganismal genome, including, but not limited to, a regulatory sequence, to a gene-encoding sequence, or to a sequence without a known role. Any of a variety of regions of microorganismal genomes that are available for modification are readily known in the art for many microorganisms, including the microorganisms specifically listed herein. As a non-limiting example, the loci of a variety of vaccinia genes provided hereinelsewhere exemplify the number of different regions that can be targeted for modification in the microorganisms provided herein. In another embodiment, the modification can be fully or partially random, whereupon selection of any particular modified microorganism can be determined according to the desired properties of the modified the microorganism.

In some embodiments, the microorganism can be modified to express an exogenous gene. Exemplary exogenous gene products include proteins and RNA molecules. The modified microorganisms can express a detectable gene product, a therapeutic gene product, a gene product for manufacturing or harvesting, or an antigenic gene product for antibody harvesting. The characteristics of such gene products are described hereinelsewhere. In some embodiments of modifying an organism to express an exogenous gene, the modification can also contain one or more regulatory sequences to regulate expression of the exogenous gene. As is known in the art, regulatory sequences can permit constitutive expression of the exogenous gene or can permit inducible expression of the exogenous gene. Further, the regulatory sequence can permit control of the level of expression of the exogenous gene. In some examples, inducible expression can be under the control of cellular or other factors present in a tumor cell or present in a microorganism-

infected tumor cell. In other examples, inducible expression can be under the control of an administerable substance, including IPTG, RU486 or other known induction compounds. Any of a variety of regulatory sequences are available to one skilled in the art according to known factors and design preferences. In some embodiments, such as gene product manufacture and harvesting, the regulatory sequence can result in constitutive, high levels of gene expression. In some embodiments, such as anti-(gene product) antibody harvesting, the regulatory sequence can result in constitutive, lower levels of gene expression. In tumor therapy embodiments, a therapeutic protein can be under the control of an internally inducible promoter or an externally inducible promoter.

In other embodiments, organ or tissue-specific expression can be controlled by regulatory sequences. In order to achieve expression only in the target organ, for example, a tumor to be treated, the foreign nucleotide sequence can be linked to a tissue specific promoter and used for gene therapy. Such promoters are well known to those skilled in the art (see *e.g.*, Zimmermann *et al.*, (1994) Neuron 12, 11-24; Vidal *et al.*; (1990) EMBO J. 9, 833-840; Mayford *et al.*, (1995), Cell 81, 891-904; Pinkert *et al.*, (1987) Genes & Dev. 1, 268-76).

In some embodiments, the microorganisms can be modified to express two or more proteins, where any combination of the two or more proteins can be one or more detectable gene products, therapeutic gene products, gene products for manufacturing or harvesting, or antigenic gene products for antibody harvesting. In one embodiment, a microorganism can be modified to express a detectable protein and a therapeutic protein. In another embodiment, a microorganism can be modified to express two or more gene products for detection or two or more therapeutic gene products. For example, one or more proteins involved in biosynthesis of a luciferase substrate can be expressed along with luciferase. When two or more exogenous genes are introduced, the genes can be regulated under the same or different regulatory sequences, and the genes can be inserted in the same or different regions of the microorganismal genome, in a single or a plurality of genetic manipulation steps. In some embodiments, one gene, such as a gene encoding a detectable gene

product, can be under the control of a constitutive promoter, while a second gene, such as a gene encoding a therapeutic gene product, can be under the control of an inducible promoter. Methods for inserting two or more genes in to a microorganism are known in the art and can be readily performed for a wide variety of microorganisms using a wide variety of exogenous genes, regulatory sequences, and/or other nucleic acid sequences.

In an example of performing microorganismal modification methods, vaccinia virus strain LIVP was modified to contain insertions of exogenous DNA in three different locations of the viral genome. Using general methods known in the art, known molecular biology tools, and sequences known in the art or disclosed herein can be used to create modified vaccinia virus strains, including viruses containing insertions in the F3 gene, TK gene and/or HA gene. See, *e.g.*, Mikryukov, *et al.*, Biotekhnologya 4 (1998), 442-449; Goebel *et al.*, Virology 179 (1990), 247-266; Antoine *et al.*, Virology 244 (1998), 365-396; Mayr *et al.*, Zentbl. Bakteriol. Hyg. Abt 1 Orig. B 167 (1978), 375-390; Ando and Matumoto, Jpn. J. Microbial. 14 (1979), 181-186; Sugimoto *et al.*, Microbial. Immuol. 29 (1985), 421-428; Takahashi-Nishimaki *et al.*, J. Gen. Virol. 68 (1987), 2705-2710). These methods include, for example, in vitro recombination techniques, synthetic methods and in vivo recombination methods as described, for example, in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, cold Spring Harbor NY (1989), and in the Examples disclosed herein. The person skilled in the art can isolate the gene encoding the gene product of F3 (or a related gene product) from any vaccinia strain using, for example, the nucleotide sequence of the F3 gene of SEQ ID No:1 or SEQ ID NOs:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 or 32, or a fragment thereof as a probe for screening a library.

Methods of producing recombinant microorganisms are known in the art. Provided herein for exemplary purposes are methods of producing a recombinant vaccinia virus. A recombinant vaccinia virus with an insertion in the F3 gene can be prepared by the following steps: (a) generating (i) a vaccinia shuttle plasmid

containing the modified F3 gene inserted at restriction site X and (ii) a dephosphorylated wt VV (VGL) DNA digested at restriction site X; (b) transfecting host cells infected with PUV-inactivated helper VV (VGL) with a mixture of the constructs of (i) and (ii) of step a; and (c) isolating the recombinant vaccinia viruses from the transfectants. One skilled in the art knows how to perform such methods, for example by following the instructions given in Example 1 and the legend to Figure 1; see also Timiryasova *et al.*, Biotechniques 31 (2001), 534-540. In one embodiment, restriction site X is a unique restriction site. A variety of suitable host cells also are known to the person skilled in the art and include many mammalian, avian and insect cells and tissues which are susceptible for vaccinia virus infection, including chicken embryo, rabbit, hamster and monkey kidney cells, for example, HeLa cells, RK_{13}, CV-1, Vero, BSC40 and BSC-1 monkey kidney cells.

2. Screening for above characteristics

Modified microorganisms can be screened for any desired characteristics, including the characteristics described herein such as attenuated pathogenicity, reduced toxicity, preferential accumulation in tumor, increased ability to activate an immune response against tumor cells, increased immunogenicity, increased or decreased replication competence, and are able to express exogenous proteins, and combinations thereof. For example, the modified microorganisms can be screened for the ability to activate an immune response against tumor cells without aggressively killing the tumor cells. In another example, the microorganisms can be screened for expression of one or more detectable genes, including genes that can be used for imaging, or for expression of one or more genes for manufacture or harvest of the gene products and/or for harvest of antibodies against the gene products.

Any of a variety of known methods for screening for such characteristics can be performed, as demonstrated in the Examples provided herein. One Exemplary method for screening for desired characteristics includes, but is not limited to, monitoring growth, replication and/or gene expression (including expression of an exogenous gene) in cell culture or other invitro medium. The cell culture can be from any organism, and from any tissue source, and can include tumorous tissues.

Other exemplary methods for screening for desired characteristics include, but are not limited to, administering a microorganism to animal, including non-human animals such as a mouse, monkey or ape, and optionally also including humans, and monitoring the microorganism, the tumor, and or the animal; monitoring can be performed by in vivo imaging of the microorganism and/or the tumor (*e.g.*, low light imaging of microorganismal gene expression or ultrasonic tumor imaging), external monitoring of the tumor (*e.g.*, external measurement of tumor size), monitoring the animal (*e.g.*, monitoring animal weight, blood panel, antibody titer, spleen size, or liver size). Other exemplary methods for screening for desired characteristics include, but are not limited to, harvesting a non-human animal for the effects and location of the microorganism and expression by the microorganism, including methods such as harvesting a variety of organs including a tumor to determine presence of the microorganism and/or gene expression by the microorganism in the organs or tumor, harvesting of organs associated with an immune response or microorganismal clearance such as the spleen or liver, harvesting the tumor to determine tumor size and viability of tumor cells, harvesting antibodies or antibody producing cells. Such screening and monitoring methods can be used in any of a variety of combinations, as is known in art. In one embodiment, a microorganism can be screened by administering the microorganism to an animal such as a non-human animal or a human, followed by monitoring by in vivo imaging. In another embodiment, a microorganism can be screened by administering the microorganism to an animal such as a non-human animal, monitoring by in vivo imaging, and then harvesting the animal. Thus, provided herein are methods for screening a microorganism for desired characteristics by administering the microorganism to an animal such as an animal with a tumor, and monitoring the animal, tumor (if present), and/or microorganism in the animal for one or more characteristics. Also provided herein are methods for screening a microorganism for desired characteristics by administering the microorganism to a non-human animal such as a non-human animal with a tumor, harvesting the animal, and assaying the animal's organs, antibody titer, and/or tumor (if present) for one or more characteristics.

Provided herein are methods for screening a microorganism for attenuated pathogenicity or reduced toxicity, where the pathogenicity or toxicity can be determined by a variety of techniques, including, but not limited to, assessing the health state of the subject, measuring the body weight of a subject, blood or urine analysis of a subject, and monitoring tissue distribution of the microorganism within the subject; such techniques can be performed on a living subject in vivo, or can be performed post mortem. Methods also can include the ability of the microorganisms to lyse cells or cause cell death, which can be determined in vivo or in vitro.

When a subject drops below a threshold body weight, the microorganism can be considered pathogenic to the subject. Exemplary thresholds can be a drop of about 5% or more, a drop of about 10% or more, or a drop of about 15% or more in body weight relative to a reference. A body weight reference can be selected from any of a variety of references used in the art; for example, a body weight reference can be the weight of the subject prior to administration of the microorganism, the body weight reference can be a control subject having the same condition as the test subject (*e.g.*, normal or tumor-injected), where the change in weight of the control is compared to the change in weight of the test subject for the time period after administration of the microorganism.

Blood or urine analysis of the subject can indicate level of immune response, level of toxins in the subject, or other levels of stress to cells, tissues or organs of the subject such as kidneys, pancreas, liver and spleen. Levels increased above established threshold levels can indicate pathogenicity of the microorganism to the subject. Threshold levels of components of blood or urine for indicating microorganismal pathogenicity are well known in the art, and any such thresholds can be selected herein according to the desired tolerance of pathogenicity or toxicity of the microorganism.

Tissue distribution of a microorganism in a subject can indicate pathogenicity or toxicity of the microorganism. In one embodiment, tissue distribution of a microorganism that is not pathogenic or toxic can be mostly in tumor relative to other tissues or organs. Microorganisms located mostly in tumor

can accumulate, for example, at least about 2-fold greater, at least about 5-fold greater, at least about 10-fold greater, at least about 100-fold greater, at least about 1,000-fold greater, at least about 10,000-fold greater, at least about 100,000-fold greater, or at least about 1,000,000-fold greater, than the microorganism accumulate in any other particular organ or tissue.

Provided herein are methods for screening a microorganism for tissue distribution or accumulation, where the tissue distribution can be determined by a variety of techniques, including, but not limited to, harvesting a non-human subject, in vivo imaging a detectable gene product in subject. Harvesting can be accomplished by euthanizing the non-human subject, and determining the accumulation of microorganisms in tumor and, optionally, the accumulation in one or more additional tissues or organs. The accumulation can be determined by any of a variety of methods, including, but not limited to, detecting gene products such as detectable gene products (*e.g.*, gfp or beta galactosidase), histological or microscopic evaluation of tissue, organ or tumor samples, or measuring the number of plaque or colony forming units present in a tissue, organ or tumor sample. In one embodiment, the desired amount of tissue distribution of a microorganism can be mostly in tumor relative to other tissues or organs. Microorganisms located mostly in tumor can accumulate, for example, at least about 2-fold greater, at least about 5-fold greater, at least about 10-fold greater, at least about 100-fold greater, at least about 1,000-fold greater, at least about 10,000-fold greater, at least about 100,000-fold greater, or at least about 1,000,000-fold greater, than the microorganism accumulate in any other particular organ or tissue.

Also provided herein are methods of screening for microorganisms that can elicit an immune response, where the immune response can be against the tumor cells or against the microorganisms. A variety of methods for measuring the ability to elicit an immune response are known in the art, and include measuring an overall increase in immune activity in a subject, measuring an increase in anti-microorganism or anti-tumor antibodies in a subject, testing the ability of a microorganism-treated (typically a non-human) subject to prevent later

infection/tumor formation or to rapidly eliminate microorganisms or tumor cells. Methods also can include the ability of the microorganisms to lyse cells or cause cell death, which can be determined in vivo or in vitro.

Also provided herein are methods for determining increased or decreased replication competence, by monitoring the speed of replication of the microorganisms. Such measurements can be performed in vivo or in vitro. For example, the speed of replication in a cell culture can be used to determine replication competence of a microorganism. In another example, the speed of replication in a tissue, organ or tumor in a subject can be used to measure replication competence. In some embodiments, decreased replication competence in non-tumor tissues and organs can be the characteristic to be selected in a screen. In other embodiments, increased replication competence in tumors can be the characteristic to be selected in a screen.

Also provided herein are methods for determining the ability of a microorganism to express genes, such as exogenous gene. Such methods can be performed in vivo or in vitro. For example, the microorganisms can be screened on selective plates for the ability to express a gene that permits survival of the microorganism or permits the microorganism to provide a detectable signal, such as turning X-gal blue. Such methods also can be performed in vivo, where expression can be determined, for example, by harvesting tissues, organs or tumors a non-human subject or by in vivo imaging of a subject.

Also provided herein are methods for determining the ability of a microorganism to express genes toward which the subject can develop antibodies, including exogenous genes toward which the subject can develop antibodies. Such methods can be performed in vivo using any of a variety of non-human subjects. For example, gene expression can be determined, for example, by bleeding a non-human subject to which a microorganism has been administered, and assaying the blood (or serum) for the presence of antibodies against the microorganism-expressed gene, or by any other method generally used for polyclonal antibody harvesting, such as production bleeds and terminal bleeds.

Also provided herein are methods for screening a microorganism that has two or more characteristics provided herein, including screening for attenuated pathogenicity, reduced toxicity, preferential accumulation in tumor, increased ability to activate an immune response against tumor cells, increased immunogenicity, increased or decreased replication competence, ability to express exogenous proteins, and ability to elicit antibody production against a microorganismally expressed gene product. A single monitoring technique, such as in vivo imaging, can be used to verify two or more characteristics, or a variety of different monitoring techniques can be used, as can be determined by one skilled in the art according to the selected characteristics and according to the monitoring techniques used.

D. Therapeutic Methods

Provided herein are therapeutic methods, including methods of treating or preventing immunoprivileged cells or tissue, including cancerous cells, tumor and metastasis. The methods provided herein include administering a microorganism to a subject containing a tumor and/or metastases. The methods provided herein do not require the microorganism to kill tumor cells or decrease the tumor size. Instead, the methods provided herein include administering to a subject a microorganism that can cause or enhance an anti-tumor immune response in the subject. In some embodiments, the microorganisms provided herein can be administered to a subject without causing microorganism-induced disease in the subject. In some embodiments, the microorganisms can accumulate in tumors or metastases. In some embodiments, the microorganisms can elicit an anti-tumor immune response in the subject, where typically the microorganism-mediated anti-tumor immune response can develop over several days, such as a week or more, 10 days or more, two weeks or more, or a month or more, as a result of little or no microorganism-cause tumor cell death. In some exemplary methods, the microorganism can be present in the tumor, and can cause an anti-tumor immune response without the microorganism itself causing enough tumor cell death to prevent tumor growth.

In some embodiments, provided herein are methods for eliciting or enhancing antibody production against a selected antigen or a selected antigen type

in a subject, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause release of a selected antigen or selected antigen type from the tumor, resulting in antibody production against the selected antigen or selected antigen type. The administered microorganisms can posses one or more characteristics including attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenicity, replication competence, ability to express exogenous genes, and ability to elicit antibody production against a microorganismally expressed gene product.

Any of a variety of antigens can be targeted in the methods provided herein, including a selected antigen such as an exogenous gene product expressed by the microorganism, or a selected antigen type such as one or more tumor antigens release from the tumor as a result of microorganism infection of the tumor (*e.g.*, by lysis, apoptosis, secretion or other mechanism of causing antigen release from the tumor). In at least some embodiments, it can be desirable to maintain release of the selected antigen or selected antigen type over a series of days, for example, at least a week, at least ten days, at least two weeks or at least a month.

Also provided herein are methods for providing a sustained antigen release within a subject, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause sustained release of an antigen, resulting in antibody production against the antigen. The sustained release of antigen can last for several days, for example, at least a week, at least ten days, at least two weeks or at least a month. The administered microorganisms can posses one or more characteristics including attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenicity, replication competence, ability to express exogenous genes, and ability to elicit antibody production against a microorganismally expressed gene product. The sustained release of antigen can result in an immune response by the microorganism-infected host, in which the host can develop antibodies against the antigen, and/or the host can mount an immune

response against cells expressing the antigen, including an immune response against tumor cells. Thus, the sustained release of antigen can result in immunization against tumor cells. In some embodiments, the microorganism-mediated sustained antigen release-induced immune response against tumor cells can result in complete removal or killing of all tumor cells.

Also provided herein are methods for inhibiting tumor growth in a subject, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause or enhance an anti-tumor immune response. The anti-tumor immune response induced as a result of tumor or metastases-accumulated microorganisms can result in inhibition of tumor growth. The administered microorganisms can posses one or more characteristics including attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenicity, replication competence, ability to express exogenous genes, and ability to elicit antibody production against a microorganismally expressed gene product.

Also provided herein are methods for inhibiting growth or formation of a metastasis in a subject, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause or enhance an anti-tumor immune response. The anti-tumor immune response induced as a result of tumor or metastasis-accumulated microorganisms can result in inhibition of metstasis growth or formation. The administered microorganisms can posses one or more characteristics including attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenicity, replication competence, ability to express exogenous genes, and ability to elicit antibody production against a microorganismally expressed gene product.

Also provided herein are methods for decreasing the size of a tumor and/or metastasis in a subject, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause or enhance an anti-tumor immune response. The anti-tumor immune response induced

as a result of tumor or metastasis-accumulated microorganisms can result in a decrease in the size of the tumor and/or metastasis. The administered microorganisms can posses one or more characteristics including attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenicity, replication competence, ability to express exogenous genes, and ability to elicit antibody production against a microorganismally expressed gene product.

Also provided herein are methods for eliminating a tumor and/or metastasis from a subject, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause or enhance an anti-tumor immune response. The anti-tumor immune response induced as a result of tumor or metastasis-accumulated microorganisms can result in elimination of the tumor and/or metastasis from the subject. The administered microorganisms can posses one or more characteristics including attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenicity, replication competence, ability to express exogenous genes, and ability to elicit antibody production against a microorganismally expressed gene product.

Methods of reducing inhibiting tumor growth, inhibiting metastatis growth and/or formation, decreasing the size of a tumor or metastasis, eliminating a tumor or metastasis, or other tumor therapeutic methods provided herein include causing or enhancing an anti-tumor immune response in the host. The immune response of the host, being anti-tumor in nature, can be mounted against tumors and/or metastases in which microorganisms have accumulated, and can also be mounted against tumors and/or metastases in which microorganisms have not accumulated, including tumors and/or metastases that form after administration of the microorganisms to the subject. Accordingly, a tumor and/or metastasis whose growth or formation is inhibited, or whose size is decreased, or that is eliminated, can be a tumor and/or metastasis in which the microorganisms have accumulated, or also can be a tumor and/or metastasis in which the microorganisms have not accumulated. Accordingly,

provided herein are methods of reducing inhibiting tumor growth, inhibiting metastatis growth and/or formation, decreasing the size of a tumor or metastasis, eliminating a tumor or metastasis, or other tumor therapeutic methods, where the method includes administering to a subject a microorganism, where the microorganism accumulates in at least one tumor or metastasis and causes or enhances an anti-tumor immune response in the subject, and the immune response also is mounted against a tumor and/or metastasis in which the microorganism cell did not accumulate. In another embodiment, methods are provided for inhibiting or preventing recurrence of a neoplastic disease or inhibiting or preventing new tumor growth, where the methods include administering to a subject a microorganism that can accumulate in a tumor and/or metastasis, and can cause or enhance an anti-tumor immune response, and the anti-tumor immune response can inhibit or prevent recurrence of a neoplastic disease or inhibit or prevent new tumor growth.

The tumor or neoplastic disease therapeutic methods provided herein, such as methods of reducing inhibiting tumor growth, inhibiting metastatis growth and/or formation, decreasing the size of a tumor or metastasis, eliminating a tumor or metastasis, or other tumor therapeutic methods, also can include administering to a subject a microorganism that can cause tumor cell lysis or tumor cell death. Such a microorganism can be the same microorganism as the microorganism that can cause or enhance an anti-tumor immune response in the subject. Microorganisms, such as the microorganisms provided herein, can cause cell lysis or tumor cell death as a result of expression of an endogenous gene or as a result of an exogenous gene. Endogenous or exogenous genes can cause tumor cell lysis or inhibit cell growth as a result of direct or indirect actions, as is known in the art, including lytic channel formation or activation of an apoptotic pathway. Gene products, such as exogenous gene products can function to activate a prodrug to an active, cytotoxic form, resulting in cell death where such genes are expressed.

Such methods of antigen production or tumor and/or metastasis treatment can include administration of a modified microorganism described herein or a microorganism having modifications with a functional equivalence to the vaccinia

virus provided herein containing a modification of the F3 gene and the TK gene and/or the HA gene, for therapy, such as for gene therapy, for cancer gene therapy, or for vaccine therapy. Such a microorganism can be used to stimulate humoral and/or cellular immune response, induce strong cytotoxic T lymphocytes responses in subjects who may benefit from such responses. For example, the microorganism can provide prophylactic and therapeutic effects against a tumor infected by the microorganism or other infectious diseases, by rejection of cells from tumors or lesions using microorganisms that express immunoreactive antigens (Earl *et al.* (1986), Science 234, 728-831; Lathe *et al.* (1987), Nature (London) 326, 878-880), cellular tumor-associated antigens (Bernards *et al.*, (1987), Proc. Natl. Acad. Sci. USA 84, 6854-6858; Estin *et al.* (1988), Proc. Natl. Acad. Sci. USA 85, 1052-1056; Kantor *et al.* (1992), J. Natl. Cancer Inst. 84, 1084-1091; Roth *et al.* (1996), Proc. Natl. Acad. Sci. USA 93, 4781-4786) and/or cytokines (*e.g.*, IL-2, IL-12), costimulatory molecules (B7-1, B7-2) (Rao *et al.* (1996), J. Immunol. 156, 3357-3365; Chamberlain *et al.* (1996), Cancer Res. 56, 2832-2836; Oertli *et al.* (1996), J. Gen. Virol. 77, 3121-3125; Qin and Chatterjee (1996), Human Gene Ther. 7, 1853-1860; McAneny *et al.* (1996), Ann. Surg. Oncol.3, 495-500), or other therapeutic proteins.

Provided herein, solid tumors can be treated with microorganisms, such as vaccinia viruses, resulting in an enormous tumor-specific microorganism replication, which can lead to tumor protein antigen and viral protein production in the tumors. As provided herein, vaccinia virus administration to mice resulted in lysis of the infected tumor cells and a resultant release of tumor-cell-specific antigens. Continuous leakage of these antigens into the body led to a very high level of antibody titer (in approximately 7-14 days) against tumor proteins, viral proteins, and the virus encoded engineered proteins in the mice. The newly synthesized antitumor antibodies and the enhanced macrophage, neutrophils count were continuously delivered via the vasculature to the tumor and thereby provided for the recruitment of an activated immune system against the tumor. The activated immune system then eliminated the foreign compounds of the tumor including the

viral particles. This interconnected release of foreign antigens boosted antibody production and continuous response of the antibodies against the tumor proteins to function like an autoimmunizing vaccination system initiated by vaccinia viral infection and replication, followed by cell lysis, protein leakage and enhanced antibody production. Thus, the present methods can provide a complete process that can be applied to all tumor systems with immunoprivileged tumor sites as site of privileged viral, bacterial, and mammalian cell growth, which can lead to tumor elimination by the host's own immune system.

In other embodiments, methods are provided for immunizing a subject, where the methods include administering to the subject a microorganism that expresses one or more antigens against which antigens the subject will develop an immune response. The immunizing antigens can be endogenous to the microorganism, such as vaccinia antigens on a vaccinia virus used to immunize against smallpox. Or the immunizing antigens can be exogenous antigens expressed by the microorganism, such as influenza or HIV antigens expressed on a viral capsid or bacterial cell surface. Thus, the microorganisms provided herein, including the modified vaccinia viruses can be used as vaccines.

1. Administration

In performing the methods provided herein, a microorganism can be administered to a subject, including a subject having a tumor or having neoplastic cells, or a subject to be immunized. An administered microorganism can be a microorganism provided herein or any other microorganism known for administration to a subject, for example, any known microorganism known for therapeutic administration to a subject, including antigenic microorganisms such as any microorganism known to be used for vaccination. In some embodiments, the microorganism administered is a microorganism containing a characteristic such as attenuated pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, high immunogenicity, replication competence, and ability to express exogenous proteins, and combinations thereof.

a. Steps prior to administering the microorganism

In some embodiments, one or more steps can be performed prior to administration of the microorganism to the subject. Any of a variety of preceding steps can be performed, including, but not limited to diagnosing the subject with a condition appropriate for microorganismal administration, determining the immunocompetence of the subject, immunizing the subject, treating the subject with a chemotherapeutic agent, treating the subject with radiation, or surgically treating the subject.

For embodiments that include administering a microorganism to a tumor-bearing subject for therapeutic purposes, the subject has typically been previously diagnosed with a neoplastic condition. Diagnostic methods also can include determining the type of neoplastic condition, determining the stage of the neoplastic conditions, determining the size of one or more tumors in the subject, determining the presence or absence of metastatic or neoplastic cells in the lymph nodes of the subject, or determining the presence of metastases of the subject. Some embodiments of therapeutic methods for administering a microorganism to a subject can include a step of determination of the size of the primary tumor or the stage of the neoplastic disease, and if the size of the primary tumor is equal to or above a threshold volume, or if the stage of the neoplastic disease is at or above a threshold stage, a microorganism is administered to the subject. In a similar embodiment, if the size of the primary tumor is below a threshold volume, or if the stage of the neoplastic disease is at or below a threshold stage, the microorganism is not yet administered to the subject; such methods can include monitoring the subject until the tumor size or neoplastic disease stage reaches a threshold amount, and then administering the microorganism to the subject. Threshold sizes can vary according to several factors, including rate of growth of the tumor, ability of the microorganism to infect a tumor, and immunocompetence of the subject. Generally the threshold size will be a size sufficient for a microorganism to accumulate and replicate in or near the tumor without being completely removed by the host's immune system, and will typically also be a size sufficient to sustain a microorganismal infection for a time long

enough for the host to mount an immune response against the tumor cells, typically about one week or more, about ten days or more, or about two weeks or more. Exemplary threshold tumor sizes for viruses such as vaccinia viruses are at least about 100 mm^3, at least about 200 mm^3, at least about 300 mm^3, at least about 400 mm^3, at least about 500 mm^3, at least about 750 mm^3, at least about 1000 mm^3, or at least about 1500 mm^3. Threshold neoplastic disease stages also can vary according to several factors, including specific requirement for staging a particular neoplastic disease, aggressiveness of growth of the neoplastic disease, ability of the microorganism to infect a tumor or metastasis, and immunocompetence of the subject. Generally the threshold stage will be a stage sufficient for a microorganism to accumulate and replicate in a tumor or metastasis without being completely removed by the host's immune system, and will typically also be a size sufficient to sustain a microorganismal infection for a time long enough for the host to mount an immune response against the neoplastic cells, typically about one week or more, about ten days or more, or about two weeks or more. Exemplary threshold stages are any stage beyond the lowest stage (*e.g.*, Stage I or equivalent), or any stage where the primary tumor is larger than a threshold size, or any stage where metastatic cells are detected.

In other embodiments, prior to administering to the subject a microorganism, the immunocompetence of the subject can be determined. The methods of administering a microorganism to a subject provided herein can include causing or enhancing an immune response in a subject. Accordingly, prior to administering a microorganism to a subject, the ability of a subject to mount an immune response can be determined. Any of a variety of tests of immunocompetence known in the art can be performed in the methods provided herein. Exemplary immunocompetence tests can examine ABO hemagglutination titers (IgM), leukocyte adhesion deficiency (LAD), granulocyte function (NBT), T and B cell quantitation, tetanus antibody titers, salivary IgA, skin test, tonsil test, complement C3 levels, and factor B levels, and lymphocyte count. One skilled in the art can determine the desirability to administer a microorganism to a subject according to the level of

immunocompetence of the subject, according to the immunogenicity of the microorganism, and, optionally, according to the immunogenicity of the neoplastic disease to be treated. Typically, a subject can be considered immunocompetent if the skilled artisan can determine that the subject is sufficiently competent to mount an immune response against the microorganism.

In some embodiments, the subject can be immunized prior to administering to the subject a microorganism according to the methods provided herein. Immunization can serve to increase the ability of a subject to mount an immune response against the microorganism, or increase the speed at which the subject can mount an immune response against a microorganism. Immunization also can serve to decrease the risk to the subject of pathogenicity of the microorganism. In some embodiments, the immunization can be performed with an immunization microorganism that is similar to the therapeutic microorganism to be administered. For example, the immunization microorganism can be a replication-incompetent variant of the therapeutic microorganism. In other embodiments, the immunization material can be digests of the therapeutic microorganism to be administered. Any of a variety of methods for immunizing a subject against a known microorganism are known in the art and can be used herein. In one example, vaccinia viruses treated with, for example, 1 microgram of psoralen and ultraviolet light at 365 nm for 4 minutes, can be rendered replication incompetent. In another embodiment, the microorganism can be selected as the same or similar to a microorganism against which the subject has been previously immunized, *e.g.*, in a childhood vaccination.

In another embodiment, the subject can have administered thereto a microorganism without any previous steps of cancer treatment such as chemotherapy, radiation therapy or surgical removal of a tumor and/or metastases. The methods provided herein take advantage of the ability of the microorganisms to enter or localize near a tumor, where the tumor cells can be protected from the subject's immune system; the microorganisms can then proliferate in such an immunoprotected region and can also cause the release, typically a sustained release, of tumor antigens from the tumor to a location in which the subject's immune

system can recognize the tumor antigens and mount an immune response. In such methods, existence of a tumor of sufficient size or sufficiently developed immunoprotected state can be advantageous for successful administration of the microorganism to the tumor, and for sufficient tumor antigen production. If a tumor is surgically removed, the microorganisms may not be able to localize to other neoplastic cells (*e.g.*, small metastases) because such cells may not yet have matured sufficiently to create an immunoprotective environment in which the microorganisms can survive and proliferate, or even if the microorganisms can localize to neoplastic cells, the number of cells or size of the mass may be too small for the microorganisms to cause a sustained release of tumor antigens in order for the host to mount an anti-tumor immune response. Thus, for example, provided herein are methods of treating a tumor or neoplastic disease in which microorganisms are administered to a subject with a tumor or neoplastic disease without removing the primary tumor, or to a subject with a tumor or neoplastic disease in which at least some tumors or neoplastic cells are intentionally permitted to remain in the subject. In other typical cancer treatment methods such as chemotherapy or radiation therapy, such methods typically have a side effect of weakening the subject's immune system. This treatment of a subject by chemotherapy or radiation therapy can reduce the subject's ability to mount an anti-tumor immune response. Thus, for example, provided herein are methods of treating a tumor or neoplastic disease in which microorganisms are administered to a subject with a tumor or neoplastic disease without treating the subject with an immune system-weakening therapy, such as chemotherapy or radiation therapy.

In an alternative embodiment, prior to administration of a microorganism to the subject, the subject can be treated in one or more cancer treatment steps that do not remove the primary tumor or that do not weaken the immune system of the subject. A variety of more sophisticated cancer treatment methods are being developed in which the tumor can be treated without surgical removal or immune-system weakening therapy. Exemplary methods include administering a compound that decreases the rate of proliferation of the tumor or neoplastic cells without

weakening the immune system (e.g,. by administering tumor suppressor compounds or by administering tumor cell-specific compounds) or administering an angiogenesis-inhibiting compound. Thus, combined methods that include administering a microorganism to a subject can further improve cancer therapy. Thus, provided herein are methods of administering a microorganism to a subject, along with prior to or subsequent to, for example, administering a compound that slows tumor growth without weakening the subject's immune system or a compound that inhibits vascularization of the tumor.

b. Mode of administration

Any mode of administration of a microorganism to a subject can be used, provided the mode of administration permits the microorganism to enter a tumor or metastasis. Modes of administration can include, but are not limited to, intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intratumor, multipuncture (*e.g.*, as used with smallpox vaccines), inhalation, intranasal, oral, intracavity (*e.g.*, administering to the bladder via a catheter, administering to the gut by suppository or enema), aural, or ocular administration. One skilled in the art can select any mode of administration compatible with the subject and the microorganism, and that also is likely to result in the microorganism reaching tumors and/or metastases. The route of administration can be selected by one skilled in the art according to any of a variety of factors, including the nature of the disease, the kind of tumor, and the particular microorganism contained in the pharmaceutical composition. Administration to the target site can be performed, for example, by ballistic delivery, as a colloidal dispersion system, or systemic administration can be performed by injection into an artery.

c. Dosage

The dosage regimen can be any of a variety of methods and amounts, and can be determined by one skilled in the art according to known clinical factors. As is known in the medical arts, dosages for any one patient can depend on many factors, including the subject's species, size, body surface area, age, sex, immunocompetence, and general health, the particular microorganism to be

administered, duration and route of administration, the kind and stage of the disease, for example, tumor size, and other compounds such as drugs being administered concurrently. In addition to the above factors, such levels can be affected by the infectivity of the microorganism, and the nature of the microorganism, as can be determined by one skilled in the art. At least some of the viruses used the in the methods provided herein can be more infectious than the bacteria used herein. Thus, in some embodiments of the present methods, virus can be administered at lower levels than bacteria. In the present methods, appropriate minimum dosage levels of microorganisms can be levels sufficient for the microorganism to survive, grow and replicate in a tumor or metastasis. Exemplary minimum levels for administering a virus to a 65 kg human can include at least about 5×10^5 plaque forming units (pfu), at least about 1×10^6 pfu, at least about 5×10^6 pfu, at least about 1×10^7 pfu, or at least about 1×10^8 pfu. Exemplary minimum levels for administering a bacterium to a 65 kg human can include at least about 5×10^6 colony forming units (cfu), at least about 1×10^7 cfu, at least about 5×10^7 cfu, at least about 1×10^8 cfu, or at least about 1×10^9 cfu. In the present methods, appropriate maximum dosage levels of microorganisms can be levels that are not toxic to the host, levels that do not cause splenomegaly of 3x or more, levels that do not result in colonies or plaques in normal tissues or organs after about 1 day or after about 3 days or after about 7 days. Exemplary maximum levels for administering a virus to a 65 kg human can include no more than about 5×10^{10} pfu, no more than about 1×10^{10} pfu, no more than about 5×10^9 pfu, no more than about 1×10^9 pfu, or no more than about 1×10^8 pfu. Exemplary maximum levels for administering a bacterium to a 65 kg human can include no more than about 5×10^{11} pfu, no more than about 1×10^{11} pfu, no more than about 5×10^{10} pfu, no more than about 1×10^{10} pfu, or no more than about 1×10^9 pfu.

d. Number of administrations

The methods provided herein can include a single administration of a microorganism to a subject or multiple administrations of a microorganism to a subject. In some embodiments, a single administration is sufficient to establish a

microorganism in a tumor, where the microorganism can proliferate and can cause or enhance an anti-tumor response in the subject; such methods do not require additional administrations of a microorganism in order to cause or enhance an anti-tumor response in a subject, which can result, for example in inhibition of tumor growth, inhibition of metastasis growth or formation, reduction in tumor or metastasis size, elimination of a tumor or metastasis, inhibition or prevention of recurrence of a neoplastic disease or new tumor formation, or other cancer therapeutic effects. In other embodiments, a microorganism can be administered on different occasions, separated in time typically by at least one day. Separate administrations can increase the likelihood of delivering a microorganism to a tumor or metastasis, where a previous administration may have been ineffective in delivering a microorganism to a tumor or metastasis. Separate administrations can increase the locations on a tumor or metastasis where microorganism proliferation can occur or can otherwise increase the titer of microorganism accumulated in the tumor, which can increase the scale of release of antigens or other compounds from the tumor in eliciting or enhancing a host's anti-tumor immune response, and also can, optionally, increase the level of microorganism-based tumor lysis or tumor cell death. Separate administrations of a microorganism can further extend a subject's immune response against microorganismal antigens, which can extend the host's immune response to tumors or metastases in which microorganisms have accumulated, and can increase the likelihood of a host mounting an anti-tumor immune response.

When separate administrations are performed, each administration can be a dosage amount that is the same or different relative to other administration dosage amounts. In one embodiment, all administration dosage amounts are the same. In other embodiments, a first dosage amount can be a larger dosage amount than one or more subsequent dosage amounts, for example, at least 10x larger, at least 100x larger, or at least 1000x larger than subsequent dosage amounts. In one example of a method of separate administrations in which the first dosage amount is greater than

one or more subsequent dosage amounts, all subsequent dosage amounts can be the same, smaller amount relative to the first administration.

Separate administrations can include any number of two or more administrations, including two, three, four, five or six administrations. One skilled in the art can readily determine the number of adminstrations to perform or the desirability of performing one or more additional administrations according to methods known in the art for monitoring therapeutic methods and other monitoring methods provided herein. Accordingly, the methods provided herein include methods of providing to the subject one or more administrations of a microorganism, where the number of administrations can be determined by monitoring the subject, and, based on the results of the monitoring, determining whether or not to provide one or more additional administrations. Deciding of whether or not to provide one or more additional administrations can be based on a variety of monitoring results, including, but not limited to, indication of tumor growth or inhibition of tumor growth, appearance of new metastases or inhibition of metastasis, the subject's anti-microorganism antibody titer, the subject's anti-tumor antibody titer, the overall health of the subject, the weight of the subject, the presence of microorganism solely in tumor and/or metastases, the presence of microorganism in normal tissues or organs.

The time period between administrations can be any of a variety of time periods. The time period between administrations can be a function of any of a variety of factors, including monitoring steps, as described in relation to the number of administrations, the time period for a subject to mount an immune response, the time period for a subject to clear microorganism from normal tissue, or the time period for microorganismal proliferation in the tumor or metastasis. In one example, the time period can be a function of the time period for a subject to mount an immune response; for example, the time period can be more than the time period for a subject to mount an immune response, such as more than about one week, more than about ten days, more than about two weeks, or more than about a month; in another example, the time period can be less than the time period for a subject to

mount an immune response, such as less than about one week, less than about ten days, less than about two weeks, or less than about a month. In another example, the time period can be a function of the time period for a subject to clear microorganism from normal tissue; for example, the time period can be more than the time period for a subject to clear microorganism from normal tissue, such as more than about a day, more than about two days, more than about three days, more than about five days, or more than about a week. In another example, the time period can be a function of the time period for microorganismal proliferation in the tumor or metastasis; for example, the time period can be more than the amount of time for a detectable signal to arise in a tumor or metastasis after administration of a microorganism expressing a detectable marker, such as about 3 days, about 5 days, about a week, about ten days, about two weeks, or about a month.

e. Co-administrations

Also provided are methods in which an additional therapeutic substance, such as a different therapeutic microorganism or a therapeutic compound is administered. These can be administered simultaneously, sequentially or intermittently with the first microorganism. The additional therapeutic substance can interact with the microorganism or a gene product thereof, or the additional therapeutic substance can act independently of the microorganism.

i. Administration of a plurality of microorganisms

Methods are provided for administering to a subject two or more microorganisms. Administration can be effected simultaneously, sequentially or intermittently. The plurality of microorganisms can be administered as a single composition or as two or more compositions. The two or more microorganisms can include at least two bacteria, at least two viruses, at least two eukaryotic cells, or two or more selected from among bacteria, viruses and eukaryotic cells. The plurality of microorganisms can be provided as combinations of compositions containing and/or as kits that include the microorganisms packaged for administration and optionally including instructions therefore. The compositions can contain the

microorganisms formulated for single dosage administration (*i.e.*, for direct administration) can require dilution or other additions.

In one embodiment, at least one of the microorganisms is a modified microorganism such as those provided herein, having a characteristic such as low pathogenicity, low toxicity, preferential accumulation in tumor, ability to activate an immune response against tumor cells, immunogenic, replication competent, ability to express exogenous proteins, and combinations thereof. The microorganisms can be administered at approximately the same time, or can be administered at different times. The microorganisms can be administered in the same composition or in the same administration method, or can be administered in separate composition or by different administration methods.

In one example, a bacteria and a virus can be administered to a subject. The bacteria and virus can be administered at the same time, or at different times. For example, the virus can be administered prior to administering the bacteria, or the bacteria can be administered prior to administering the virus; typically the virus is administered prior to administering the bacteria. As provided herein, administering to a subject a virus prior to administering to the subject a bacterium can increase the amount of bacteria that can accumulate and/or proliferate in a tumor, relative to methods in which bacteria alone are administered.

Accordingly, the methods provided herein that include administration of virus prior to administration of bacteria permit the administration of a lower dosage amount of bacteria than would otherwise be administered in a method in which bacteria alone are administered or a method in which bacteria are administered at the same time as or prior to administration of a virus. For example, in some embodiments, a bacterium to be administered can have one or more properties that limit the ability of the bacterium to be used, such properties can include, but are not limited to toxicity, low tumor specificity of accumulation, and limited proliferation capacity. A bacterium to be administered that has one or more limiting properties can require administration in lower dosage amounts, or can require assistance in tumor-specific accumulation and/or proliferation. Provided herein are methods of

administering such a bacterium with limiting properties, where prior to administering the bacterium, a virus is administered such that the limited bacterium can be administered in smaller quantities, can accumulate in tumor with increased specificity, and/or can have an increased ability to proliferate in a tumor.

The time period between administrations can be any time period that achieves the desired effects, as can be determined by one skilled in the art. Selection of a time period between administrations of different microorganisms can be determined according to parameters similar to those for selecting the time period between administrations of the same microorganism, including results from monitoring steps, the time period for a subject to mount an immune response, the time period for a subject to clear microorganism from normal tissue, or the time period for microorganismal proliferation in the tumor or metastasis. In one example, the time period can be a function of the time period for a subject to mount an immune response; for example, the time period can be more than the time period for a subject to mount an immune response, such as more than about one week, more than about ten days, more than about two weeks, or more than about a month; in another example, the time period can be less than the time period for a subject to mount an immune response, such as less than about one week, less than about ten days, less than about two weeks, or less than about a month. In another example, the time period can be a function of the time period for a subject to clear microorganism from normal tissue; for example, the time period can be more than the time period for a subject to clear microorganism from normal tissue, such as more than about a day, more than about two days, more than about three days, more than about five days, or more than about a week. In another example, the time period can be a function of the time period for microorganismal proliferation in the tumor or metastasis; for example, the time period can be more than the amount of time for a detectable signal to arise in a tumor or metastasis after administration of a microorganism expressing a detectable marker, such as about 3 days, about 5 days, about a week, about ten days, about two weeks, or about a month. In one example a virus can first be administered, and a bacteria can be administered about 5 days after

administration of the virus. In another example, a virus can be first administered, and a bacterium can be administered upon detection of a virally-encoded detectable gene product in the tumor of the subject, optionally when the virally-encoded detectable gene product is detected only in the tumor of the subject.

ii. Therapeutic compounds

The methods can include administering one or more therapeutic compounds to the subject in addition to administering a microorganism or plurality thereof to a subject. Therapeutic compounds can act independently, or in conjunction with the microorganism, for tumor therapeutic effects. Therapeutic compounds that can act independently include any of a variety of known chemotherapeutic compounds that can inhibit tumor growth, inhibit metastasis growth and/or formation, decrease the size of a tumor or metastasis, eliminate a tumor or metastasis, without reducing the ability of a microorganism to accumulate in a tumor, replicate in the tumor, and cause or enhance an anti-tumor immune response in the subject.

Therapeutic compounds that act in conjunction with the microorganisms include, for example, compounds that alter the expression of the microorganism or compounds that can interact with a microorganism-expressed gene, or compounds that can inhibit microorganismal proliferation, including compounds toxic to the microorganism. Therapeutic compounds that can act in conjunction with the microorganism include, for example, therapeutic compounds that increase the proliferation, toxicity, tumor cell killing, or immune response eliciting properties of a microorganism, and also can include, for example, therapeutic compounds that decrease the proliferation, toxicity, or cell killing properties of a microorganism. Thus, provided herein are methods of administering to a subject one or more therapeutic compounds that can act in conjunction with the microorganism to increase the proliferation, toxicity, tumor cell killing, or immune response eliciting properties of a microorganism. Also provided herein are methods of administering to a subject one or more therapeutic compounds that can act in conjunction with the microorganism to decrease the proliferation, toxicity, or cell killing properties of a microorganism.

In one embodiment, therapeutic compounds that can act in conjunction with the microorganism to increase the proliferation, toxicity, tumor cell killing, or immune response eliciting properties of a microorganism are compounds that can alter gene expression, where the altered gene expression can result in an increased killing of tumor cells or an increased anti-tumor immune response in the subject. A gene expression-altering compound can, for example, cause an increase or decrease in expression of one or more microorganismal genes, including endogenous microorganismal genes and/or exogenous microorganismal genes. For example, a gene expression-altering compound can induce or increase transcription of a gene in a microorganism such as an exogenous gene that can cause cell lysis or cell death, that can provoke an immune response, that can catalyze conversion of a prodrug-like compound, or that can inhibit expression of a tumor cell gene. Any of a wide variety of compounds that can alter gene expression are known in the art, including IPTG and RU486. Exemplary genes whose expression can be up-regulated include proteins and RNA molecules, including toxins, enzymes that can convert a prodrug to an anti-tumor drug, cytokines, transcription regulating proteins, siRNA, and ribozymes. In another example, a gene expression-altering compound can inhibit or decrease transcription of a gene in a microorganism such as an exogenous gene that can reduce microorganismal toxicity or reduces microorganismal proliferation. Any of a variety of compounds that can reduce or inhibit gene expression can be used in the methods provided herein, including siRNA compounds, transcriptional inhibitors or inhibitors of transcriptional activators. Exemplary genes whose expression can be down-regulated include proteins and RNA molecules, including microorganismal proteins or RNA that suppress lysis, nucleotide synthesis or proliferation, and cellular proteins or RNA molecules that suppress cell death, immunoreactivity, lysis, or microorganismal replication.

In another embodiment, therapeutic compounds that can act in conjunction with the microorganism to increase the proliferation, toxicity, tumor cell killing, or immune response eliciting properties of a microorganism are compounds that can interact with a microorganism-expressed gene product, and such interaction can

result in an increased killing of tumor cells or an increased anti-tumor immune response in the subject. A therapeutic compound that can interact with a microorganism-expressed gene product can include, for example a prodrug or other compound that has little or no toxicity or other biological activity in its subject-administered form, but after interaction with a microorganism-expressed gene product, the compound can develop a property that results in tumor cell death, including but not limited to, cytotoxicity, ability to induce apoptosis, or ability to trigger an immune response. A variety of prodrug-like substances are known in the art and an exemplary set of such compounds are disclosed elsewhere herein, where such compounds can include gancyclovir, 5-fluorouracil, 6-methylpurine deoxyriboside, cephalosporin-doxorubicin, 4-[(2-chloroethyl)(2-mesuloxyethyl)amino]benzoyl-L-glutamic acid, acetominophen, indole-3-acetic acid, CB1954, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, bis-(2-chloroethyl)amino-4-hydroxyphenylaminomethanone 28, 1-chloromethyl-5-hydroxyl-1,2-dihyro-3H-benz[e]indole, epirubicin-glucuronide, 5'-deoxy5-fluorouridine, cytosine arabinoside, and linamarin.

In another embodiment, therapeutic compounds that can act in conjunction with the microorganism to decrease the proliferation, toxicity, or cell killing properties of a microorganism are compounds that can inhibit microorganismal replication, inhibit microorganismal toxins, or cause microorganismal death. A therapeutic compound that can inhibit microorganismal replication, inhibit microorganismal toxins, or cause microorganismal death can generally include a compound that can block one or more steps in the microorganismal life cycle, including, but not limited to, compounds that can inhibit microorganismal DNA replication, microorganismal RNA transcription, viral coat protein assembly, outer membrane or polysaccharide assembly. Any of a variety of compounds that can block one or more steps in a microorganismal life cycle are known in the art, including any known antibiotic, microorganismal DNA polymerase inhibitors, microorganismal RNA polymerase inhibitors, inhibitors of proteins that regulate microorganismal DNA replication or RNA transcription. In one example, when a

microorganism is a bacteria, a compound can be an antibiotic. In another example, a microorganism can contain a gene encoding a microorganismal life cycle protein, such as DNA polymerase or RNA polymerase that can be inhibited by a compound that is, optionally, non-toxic to the host organism.

f. State of subject

In another embodiment, the methods provided herein for administering a microorganism to a subject can be performed on a subject in any of a variety of states, including an anesthetized subject, an alert subject, a subject with elevated body temperature, a subject with reduced body temperature, or other state of the subject that is known to affect the accumulation of microorganism in the tumor. As provided herein, it has been determined that a subject that is anesthetized can have a decreased rate of accumulation of a microorganism in a tumor relative to a subject that is not anesthetized. Further provided herein, it has been determined that a subject with decreased body temperature can have a decreased rate o accumulation of a microorganism in a tumor relative to a subject with a normal body temperature. Accordingly, provided herein are methods of administering a microorganism to a subject, where the methods can include administering a microorganism to a subject where the subject is not under anesthesia, such as general anesthesia; for example, the subject can be under local anesthesia, or can be unanesthetized. Also provided herein are methods of administering a microorganism to a subject, where the methods can include administering a microorganism to a subject with altered body temperature, where the alteration of the body temperature can influence the ability of the microorganism to accumulate in a tumor; typically, a decrease in body temperature can decrease the ability of a microorganism to accumulate in a tumor. Thus, in one exemplary embodiment, a method is provided for administering a microorganism to a subject, where the method includes elevating the body temperature of the subject to a temperature above normal, and administering a microorganism to the subject, where the microorganism can accumulate in the tumor more readily in the subject with higher body temperature relative to the ability of the

microorganism to accumulate in a tumor of a subject with a normal body temperature.

2. Monitoring

The methods provided herein can further include one or more steps of monitoring the subject, monitoring the tumor, and/or monitoring the microorganism administered to the subject. Any of a variety of monitoring steps can be included in the methods provided herein, including, but not limited to, monitoring tumor size, monitoring anti-(tumor antigen) antibody titer, monitoring the presence and/or size of metastases, monitoring the subject's lymph nodes, monitoring the subject's weight or other health indicators including blood or urine markers, monitoring anti-(microorganismal antigen) antibody titer, monitoring microorganismal expression of a detectable gene product, and directly monitoring microorganismal titer in a tumor, tissue or organ of a subject.

The purpose of the monitoring can be simply for assessing the health state of the subject or the progress of therapeutic treatment of the subject, or can be for determining whether or not further administration of the same or a different microorganism is warranted, or for determining when or whether or not to administer a compound to the subject where the compound can act to increase the efficacy of the therapeutic method, or the compound can act to decrease the pathogenicity of the microorganism administered to the subject.

a. Monitoring microorganismal gene expression

In some embodiments, the methods provided herein can include monitoring one or more microorganismally expressed genes. Microorganisms, such as those provided herein or otherwise known in the art, can express one or more detectable gene products, including but not limited to, detectable proteins.

As provided herein, measurement of a detectable gene product expressed in a microorganism can provide an accurate determination of the level of microorganism present in the subject. As further provided herein, measurement of the location of the detectable gene product, for example, by imaging methods including tomographic methods, can determine the localization of the microorganism in the

subject. Accordingly, the methods provided herein that include monitoring a detectable microorganismal gene product can be used to determine the presence or absence of the microorganism in one or more organs or tissues of a subject, and/or the presence or absence of the microorganism in a tumor or metastases of a subject. Further, the methods provided herein that include monitoring a detectable microorganismal gene product can be used to determine the titer of microorganism present in one or more organs, tissues, tumors or metastases. Methods that include monitoring the localization and/or titer of microorganisms in a subject can be used for determining the pathogenicity of a microorganism; since microorganismal infection, and particularly the level of infection, of normal tissues and organs can indicate the pathogenicity of the probe, methods of monitoring the localization and/or amount of microorganisms in a subject can be used to determine the pathogenicity of a microorganism. Since methods provided herein can be used to monitor the amount of microorganisms at any particular location in a subject, the methods that include monitoring the localization and/or titer of microorganisms in a subject can be performed at multiple time points, and, accordingly can determine the rate of microorganismal replication in a subject, including the rate of microorganismal replication in one or more organs or tissues of a subject; accordingly, the methods of monitoring a microorganismal gene product can be used for determining the replication competence of a microorganism. The methods provided herein also can be used to quantitate the amount of microorganism present in a variety of organs or tissues, and tumors or metastases, and can thereby indicate the degree of preferential accumulation of the microorganism in a subject; accordingly, the microorganismal gene product monitoring methods provided herein can be used in methods of determining the ability of a microorganism to accumulate in tumor or metastases in preference to normal tissues or organs. Since the microorganisms used in the methods provided herein can accumulate in an entire tumor or can accumulate at multiple sites in a tumor, and can also accumulate in metastases, the methods provided herein for monitoring a microorganismal gene product can be used to determine the size of a tumor or the number of metastases are

present in a subject. Monitoring such presence of microorganismal gene product in tumor or metastasis over a range of time can be used to assess changes in the tumor or metastasis, including growth or shrinking of a tumor, or development of new metastases or disappearance of metastases, and also can be used to determine the rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases, or the change in the rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases. Accordingly, the methods of monitoring a microorganismal gene product can be used for monitoring a neoplastic disease in a subject, or for determining the efficacy of treatment of a neoplastic disease, by determining rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases, or the change in the rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases.

Any of a variety of detectable proteins can be detected in the monitoring methods provided herein; an exemplary, non-limiting list of such detectable proteins includes any of a variety of fluorescence proteins (*e.g.*, green fluorescence proteins), any of a variety of luciferases, transferring or other iron binding proteins; or receptors, binding proteins, and antibodies, where a compound that specifically binds the receptor, binding protein or antibody can be a detectable agent or can be labeled with a detectable substance (*e.g.*, a radionuclide or imaging agent).

b. Monitoring tumor size

Also provided herein are methods of monitoring tumor and/or metastasis size and location. Tumor and or metastasis size can be monitored by any of a variety of methods known in the art, including external assessment methods or tomographic or magnetic imaging methods. In addition to the methods known in the art, methods provided herein, for example, monitoring microorganismal gene expression, can be used for monitoring tumor and/or metastasis size.

Monitoring size over several time points can provide information regarding the increase or decrease in size of a tumor or metastasis, and can also provide information regarding the presence of additional tumors and/or metastases in the

subject. Monitoring tumor size over several time points can provide information regarding the development of a neoplastic disease in a subject, including the efficacy of treatment of a neoplastic disease in a subject.

c. Monitoring antibody titer

The methods provided herein also can include monitoring the antibody titer in a subject, including antibodies produced in response to administration of a microorganism to a subject. The microorganisms administered in the methods provided herein can elicit an immune response to endogenous microorganismal antigens. The microorganisms administered in the methods provided herein also can elicit an immune response to exogenous genes expressed by a microorganism. The microorganisms administered in the methods provided herein also can elicit an immune response to tumor antigens. Monitoring antibody titer against microorganismal antigens, microorganismally expressed exogenous gene products, or tumor antigens can be used in methods of monitoring the toxicity of a microorganism, monitoring the efficacy of treatment methods, or monitoring the level of gene product or antibodies for production and/or harvesting.

In one embodiment, monitoring antibody titer can be used to monitor the toxicity of a microorganism. Antibody titer against a microorganism can vary over the time period after administration of the microorganism to the subject, where at some particular time points, a low anti-(microorganismal antigen) antibody titer can indicate a higher toxicity, while at other time points a high anti-(microorganismal antigen) antibody titer can indicate a higher toxicity. The microorganisms used in the methods provided herein can be immunogenic, and can, therefore, elicit an immune response soon after administering the microorganism to the subject. Generally, a microorganism against which a subject's immune system can quickly mount a strong immune response can be a microorganism that has low toxicity when the subject's immune system can remove the microorganism from all normal organs or tissues. Thus, in some embodiments, a high antibody titer against microorganismal antigens soon after administering the microorganism to a subject can indicate low toxicity of a microorganism. In contrast, a microorganism that is

not highly immunogenic may infect a host organism without eliciting a strong immune response, which can result in a higher toxicity of the microorganism to the host. Accordingly, in some embodiments, a high antibody titer against microorganismal antigens soon after administering the microorganism to a subject can indicate low toxicity of a microorganism.

In other embodiments, monitoring antibody titer can be used to monitor the efficacy of treatment methods. In the methods provided herein, antibody titer, such as anti-(tumor antigen) antibody titer, can indicate the efficacy of a therapeutic method such as a therapeutic method to treat neoplastic disease. Therapeutic methods provided herein can include causing or enhancing an immune response against a tumor and/or metastasis. Thus, by monitoring the anti-(tumor antigen) antibody titer, it is possible to monitor the efficacy of a therapeutic method in causing or enhancing an immune response against a tumor and/or metastasis. The therapeutic methods provided herein also can include administering to a subject a microorganism that can accumulate in a tumor and can cause or enhance an anti-tumor immune response. Accordingly, it is possible to monitor the ability of a host to mount an immune response against microorganisms accumulated in a tumor or metastasis, which can indicate that a subject has also mounted an anti-tumor immune response, or can indicate that a subject is likely to mount an anti-tumor immune response, or can indicate that a subject is capable of mounting an anti-tumor immune response.

In other embodiments, monitoring antibody titer can be used for monitoring the level of gene product or antibodies for production and/or harvesting. As provided herein, methods can be used for producing proteins, RNA molecules or other compounds by expressing an exogenous gene in a microorganism that has accumulated in a tumor. Further provided herein are methods for producing antibodies against a protein, RNA molecule or other compound produced by exogenous gene expression of a microorganism that has accumulated in a tumor. Monitoring antibody titer against the protein, RNA molecule or other compound can indicate the level of production of the protein, RNA molecule or other compound by

the tumor-accumulated microorganism, and also can directly indicate the level of antibodies specific for such a protein, RNA molecule or other compound.

d. Monitoring general health diagnostics

The methods provided herein also can include methods of monitoring the health of a subject. Some of the methods provided herein are therapeutic methods, including neoplastic disease therapeutic methods. Monitoring the health of a subject can be used to determine the efficacy of the therapeutic method, as is known in the art. The methods provided herein also can include a step of administering to a subject a microorganism. Monitoring the health of a subject can be used to determine the pathogenicity of a microorganism administered to a subject. Any of a variety of health diagnostic methods for monitoring disease such as neoplastic disease, infectious disease, or immune-related disease can be monitored, as is known in the art. For example, the weight, blood pressure, pulse, breathing, color, temperature or other observable state of a subject can indicate the health of a subject. In addition, the presence or absence or level of one or more components in a sample from a subject can indicate the health of a subject. Typical samples can include blood and urine samples, where the presence or absence or level of one or more components can be determined by performing, for example, a blood panel or a urine panel diagnostic test. Exemplary components indicative of a subject's health include, but are not limited to, white blood cell count, hematocrit, c-reactive protein concentration.

e. Monitoring coordinated with treatment

Also provided herein are methods of monitoring a therapy, where therapeutic decisions can be based on the results of the monitoring. Therapeutic methods provided herein can include administering to a subject a microorganism, where the microorganism can preferentially accumulate in a tumor and/or metastasis, and where the microorganism can cause or enhance an anti-tumor immune response. Such therapeutic methods can include a variety of steps including multiple administrations of a particular microorganism, administration of a second microorganism, or administration of a therapeutic compound. Determination of the

amount, timing or type of microorganism or compound to administer to the subject can be based on one or more results from monitoring the subject. For example, the antibody titer in a subject can be used to determine whether or not it is desirable to administer a microorganism or compound, the quantity of microorganism or compound to administer, and the type of microorganism or compound to administer, where, for example, a low antibody titer can indicate the desirability of administering additional microorganism, a different microorganism, or a therapeutic compound such as a compound that induces microorganismal gene expression. In another example, the overall health state of a subject can be used to determine whether or not it is desirable to administer a microorganism or compound, the quantity of microorganism or compound to administer, and the type of microorganism or compound to administer, where, for example, determining that the subject is healthy can indicate the desirability of administering additional microorganism, a different microorganism, or a therapeutic compound such as a compound that induces microorganismal gene expression. In another example, monitoring a detectable microorganismally expressed gene product can be used to determine whether or not it is desirable to administer a microorganism or compound, the quantity of microorganism or compound to administer, and the type of microorganism or compound to administer. Such monitoring methods can be used to determine whether or not the therapeutic method is effective, whether or not the therapeutic method is pathogenic to the subject, whether or not the microorganism has accumulated in a tumor or metastasis, and whether or not the microorganism has accumulated in normal tissues or organs. Based on such determinations, the desirability and form of further therapeutic methods can be derived.

In one embodiment, determination of whether or not a therapeutic method is effective can be used to derive further therapeutic methods. Any of a variety of methods of monitoring can be used to determine whether or not a therapeutic method is effective, as provided herein or otherwise known in the art. If monitoring methods indicate that the therapeutic method is effective, a decision can be made to maintain the current course of therapy, which can include further administrations of

a microorganism or compound, or a decision can be made that no further administrations are required. If monitoring methods indicate that the therapeutic method is ineffective, the monitoring results can indicate whether or not a course of treatment should be discontinued (*e.g.*, when a microorganism is pathogenic to the subject), or changed (*e.g.*, when a microorganism accumulates in a tumor without harming the host organism, but without eliciting an anti-tumor immune response), or increased in frequency or amount (*e.g.*, when little or no microorganism accumulates in tumor).

In one example, monitoring can indicate that a microorganism is pathogenic to a subject. In such instances, a decision can be made to terminate administration of the microorganism to the subject, to administer lower levels of the microorganism to the subject, to administer a different microorganism to a subject, or to administer to a subject a compound that reduces the pathogenicity of the microorganism. In one example, administration of a microorganism that is determined to be pathogenic can be terminated. In another example, the dosage amount of a microorganism that is determined to be pathogenic can be decreased for subsequent administration; in one version of such an example, the subject can be pre-treated with another microorganism that can increase the ability of the pathogenic microorganism to accumulate in tumor, prior to re-administering the pathogenic microorganism to the subject. In another example, a subject can have administered thereto a bacteria or virus that is pathogenic to the subject; administration of such a pathogenic microorganism can be accompanied by administration of, for example an antibiotic, anti-microorganismal compound, pathogenicity attenuating compound (*e.g.*, a compound that down-regulates the expression of a lytic or apoptotic gene product), or other compound that can decrease the proliferation, toxicity, or cell killing properties of a microorganism, as described herein elsewhere. In one variation of such an example, the localization of the microorganism can be monitored, and, upon determination that the microorganism is accumulated in tumor and/or metastases but not in normal tissues or organs, administration of the antibiotic, anti-microorganismal compound or pathogenicity attenuating compound can be

terminated, and the pathogenic activity of the microorganism can be activated or increased, but limited to the tumor and/or metastasis. In another variation of such an example, after terminating administration of an antibiotic, anti-microorganismal compound or pathogenicity attenuating compound, the presence of the microorganism and/or pathogenicity of the microorganism can be further monitored, and administration of such a compound can be reinitiated if the microorganism is determined to pose a threat to the host by, for example, spreading to normal organs or tissues, releasing a toxin into the vasculature, or otherwise having pathogenic effects reaching beyond the tumor or metastasis.

In another example, monitoring can determine whether or not a microorganism has accumulated in a tumor or metastasis of a subject. Upon such a determination, a decision can be made to further administer additional microorganism, a different microorganism or a compound to the subject. In one example, monitoring the presence of a virus in a tumor or metastasis can be used in deciding to administer to the subject a bacterium, where, for example, the quantity of bacteria administered can be reduced according to the presence and/or quantity of virus in a tumor or metastasis. In a similar example, monitoring the presence of a virus in a tumor or metastasis can be used in deciding when to administer to the subject a bacterium, where, for example, the bacteria can be administered upon detecting to the presence and/or a selected quantity of virus in a tumor or metastasis. In another example, monitoring the presence of a microorganism in a tumor can be used in deciding to administer to the subject a compound, where the compound can increase the pathogenicity, proliferation, or immunogenicity of a microorganism or the compound can otherwise act in conjunction with the microorganism to increase the proliferation, toxicity, tumor cell killing, or immune response eliciting properties of a microorganism; in one variation of such an example, the microorganism can, for example have little or no lytic or cell killing capability in the absence of such a compound; in a further variation of such an example, monitoring of the presence of the microorganism in a tumor or metastasis can be coupled with monitoring the absence of the microorganism in normal tissues or organs, where the compound is

administered if the microorganism is present in tumor or metastasis and not at all present or substantially not present in normal organs or tissues; in a further variation of such an example, the amount of microorganism in a tumor or metastasis can be monitored, where the compound is administered if the microorganism is present in tumor or metastasis at sufficient levels.

E. Methods of Producing Gene Products and Antibodies

Provided herein are microorganisms, and methods for making and using such microorganisms for production products of exogenous genes and/or for production of antibodies specific for exogenous gene products. The methods provided herein result in efficient recombinant production of biologically active proteins. In EP Al 1 281 772, it is disclosed that when vaccinia virus (LIVP strain) carrying the light emitting fusion gene construct rVV-ruc-gfp was injected intravenously into nude mice, the virus particles were found to be cleared from all internal organs within 4 days, as determined by extinction of light emission. In contrast, when the fate of the injected vaccinia virus was similarly followed in nude mice bearing tumors grown from subcutaneously implanted C6 rat glioma cells, virus particles were found to be retained over time in the tumor tissues, resulting in lasting light emission. The presence and amplification of the virus-encoded fusion proteins in the same tumor were monitored in live animals by observing GFP fluorescence under a stereomicroscope and by detecting luciferase-catalyzed light emission under a low-light video-imaging camera. Tumor-specific light emission was detected 4 days after viral injection in nude mice carrying subcutaneous C6 glioma implants. Tumor accumulation of rVV-ruc-gfp virus particles was also seen in nude mice carrying subcutaneous tumors developed from implanted PC-3 human prostate cells, and in mice with orthotopically implanted MCF-7 human breast tumors. Further, intracranial C6 rat glioma cell implants in immunocompetent rats and MB-49 human bladder tumor cell implants in C57 mice were also targeted by the vaccinia virus. In addition to primary breast tumors, small metastatic tumors were also detected externally in the contralateral breast region, as well as in nodules on the exposed lung surface, suggesting metastasis to the contralateral breast and lung. In summary

it was shown that light-emitting cells or microorganisms, for example, vaccinia virus can be used to detect and treat metastatic tumors.

Similar results were obtained with light-emitting bacteria (Salmonella, Vibrio, Listeria, E. coli) which were injected intravenously into mice and which could be visualized in whole animals under a low light imager immediately. No light emission was detected twenty four hours after bacterial injection in both athymic (nu/nu) mice and immunocompetent C57 mice as a result of clearing by the immune system. In nude mice bearing tumors developed from implanted C6 glioma cells, light emission was abolished from the animal entirely twenty four hours after delivery of bacteria, similar to mice without tumors. However, forty eight hours post-injection, a strong, rapidly increasing light emission originated only from the tumor regions was observed. This observation indicated a continuous bacterial replication in the tumor tissue. The extent of light emission was dependent on the bacterial strain used. The homing-in process together with the sustained light emission was also demonstrated in nude mice carrying prostate, bladder, and breast tumors. In addition to primary tumors, metastatic tumors could also be visualized as exemplified in the breast tumor model. Tumor-specific light emission was also observed in immunocompetent C57 mice, with bladder tumors as well as in Lewis rats with brain glioma implants. Once in the tumor, the light-emitting bacteria were not observed to be released into the circulation and to re-colonize subsequently implanted tumors in the same animal. Further, mammalian cells expressing the Ruc-GFP fusion protein, upon injection into the bloodstream, were also found to home in to, and propagate in, glioma tumors. These findings opened the way for designing multifunctional viral vectors useful for the detection of tumors based on signals such as light emission, for suppression of tumor development and angiogenesis signaled by, for example, light extinction and the development of bacterial and mammalian cell-based tumor targeting systems in combination with therapeutic gene constructs for the treatment of cancer. These systems have the following advantages: (a) They target the tumor specifically without affecting normal tissue; (b) the expression and secretion of the therapeutic gene constructs can be, optionally, under the control of

an inducible promoter enabling secretion to be switched on or off; and (c) the location of the delivery system inside the tumor can be verified by direct visualization before activating gene expression and protein delivery.

As provided herein, the system described above based on the accumulation of bacteria, viruses and eukaryotic cells in tumors can be used for simple, quick, and inexpensive production of proteins and other biological compounds originating from cloned nucleotide sequences. This system also is useful for the concomitant overproduction of polypeptides, RNA or other biological compounds (in tumor tissue) and antibodies against those compounds (in the serum) in the same animal. As provided herein, after intravenous injection, a microorganism such as vaccinia virus can enter the tumor of an animal and, due to the immunoprivileged state of the tumor, can replicate preferentially in the tumor tissues and thereby can overproduce the inserted gene encoded protein in the tumors. After harvesting the tumor tissues, the localized and overexpressed protein can be isolated by a simple procedure from tumor homogenates. In addition, based on the findings that only 0.2 to 0.3% of the desired proteins produced in the tumor were found in the blood stream of the same animal, a simultaneous vaccination of the mouse and efficient antibody production against the overproduced protein was achieved. Thus, serum from the same mouse (or any other animal) can be harvested and used as mouse-derived antibodies against the proteins or other products overproduced in the tumor.

Thus, provided herein are methods of producing gene products and or antibodies in a non-human subject, by administering to a subject containing a tumor, a microorganism, where the microorganism expresses a selected protein or RNA to be produced, a protein or RNA whose expression can result in the formation of a compound to be produced, or a selected protein or RNA against which an antibody is to be produced. The methods provided herein can further include administering to a subject containing a tumor, a microorganism expressing an exogenous gene encoding a selected protein or RNA to be produced, a protein or RNA whose expression can result in the formation of a compound to be produced, or a selected protein or RNA against which an antibody is to be produced. The methods provided

herein can further include administering to a subject containing a tumor, a microorganism expressing an gene encoding a selected protein or RNA to be produced, a protein or RNA whose expression can result in the formation of a compound to be produced, or a selected protein or RNA against which an antibody is to be produced, where such gene expression can be regulated, for example, by a transcriptional activator or inducer, or a transcriptional suppressor. The methods provided herein for producing a protein, RNA, compound or antibody can further include monitoring the localization and/or level of the microorganism in the subject by detecting a detectable protein, where the detectable protein can indicate the expression of the selected gene, or can indicate the readiness of the microorganism to be induced to express the selected gene or for suppression of expression to be terminated or suspended. Also provided herein are methods of producing gene products and or antibodies in a non-human subject, by administering to a subject containing a tumor, a microorganism, where the microorganism expresses a selected protein or RNA to be produced, a protein or RNA whose expression can result in the formation of a compound to be produced, or a selected protein or RNA against which an antibody is to be produced, where the subject to which the microorganism is administered is not a transgenic animal. Also provided herein are methods of producing gene products and or antibodies in a non-human subject, by administering to a subject containing a tumor, a microorganism, where the microorganism expresses a selected protein to be produced, where the tumor within the subject is selected according to its ability to post-translationally process the selected protein.

The advantages of the system, include:

(a) No production of a transgenic animal carrying the novel polypeptide-encoding cassette is required;

(b) the tumor system is more efficient than tissue culture;

(c) proteins interfering with animal development and other toxic proteins can be overproduced in tumors without negative effects to the host animal;

(d) the system is fast: within 4 to 6 weeks from cDNA cloning to protein and antisera purification;

(e) the system is relatively inexpensive and can be scaled up easily;

(f) correct protein folding and modifications can be achieved;

(g) high antigenicity can be achieved, which is beneficial for better antibody production; and

(h) species-specific-cell-based production of proteins in animals such as mice, with tumors as fermentors can be achieved.

Depiction of an exemplary method for production of gene products and/or antibodies against gene products is provided in Figure 2.

In one embodiment, methods are provided for producing a desired polypeptide, RNA or compound, the method including the following steps: (a) injecting a microorganism containing a nucleotide sequence encoding the desired polypeptide or RNA into an animal bearing a tumor; (b) harvesting the tumor tissue from the animal; and (c) isolating the desired polypeptide, RNA or compound from the tumor tissue.

Steps of an exemplary method can be summarized as follows (shown for a particular embodiment, *i.e.* vaccinia virus additionally containing a gene encoding a light-emitting protein):

(1) Insertion of the desired DNA or cDNA into the vaccinia virus genome;

(2) modification of the vaccinia virus genome with light-emitting protein construct as expression marker;

(3) recombination and virus assembly in cell culture;

(4) screening of individual viral particles carrying inserts followed by large scale virus production and concentration;

(5) administration of the viral particles into mice or other animals bearing tumors of human, non-human primate or other mammalian origins;

(6) verification of viral replication and protein overproduction in animals based on light emission;

(7) harvest of tumor tissues and, optionally, the blood (separately); and

(8) purification of overexpressed proteins from tumors and, optionally, antisera from blood using conventional methods.

Any microorganism can be used in the methods provided herein, provided that they replicate in the animal, are not pathogenic for the animal, for example, are attenuated, and are recognized by the immune system of the animal. In some embodiments, such microorganisms also can express exogenous genes. Suitable microorganisms and cells are, for example, disclosed in EP A1 1 281 772 and EP A1 1 281 767. The person skilled in the art also knows how to generate animals carrying the desired tumor (see, *e.g.*, EP A1 1 281 767 or EP A1 1 281 777).

Also provide is a method for simultaneously producing a desired polypeptide, RNA or compound and an antibody directed to the polypeptide, RNA or compound, the method having the following steps: (a) administering a microorganism containing a nucleotide sequence encoding the desired polypeptide or RNA into an animal bearing a tumor; (b) harvesting the tumor tissue from the animal; (c) isolating the desired polypeptide, RNA or compound from the tumor tissue; and (d) isolating the antibody directed to the polypeptide, RNA or compound from the serum obtained from the animal. This approach can be used for generating polypeptides and/or antibodies against the polypeptides which are toxic or unstable, or which require species specific cellular environment for correct folding or modifications.

In another embodiment, the microorganism can further contain a nucleotide sequence encoding a detectable protein, such as a luminescent or fluorescent protein, or a protein capable of inducing a detectable signal.

Typically in methods for transfecting the microorganisms or cells with nucleotide sequences encoding the desired polypeptide or RNA and, optionally, a nucleotide sequence encoding a detectable protein such as a luminescent or fluorescent protein, or a protein capable of inducing a detectable signal, the nucleotide sequences are present in a vector or an expression vector. A person skilled in the art is familiar with a variety of expression vectors, which can be selected according to the microorganism used to infect the tumor, the cell type of the tumor, the organism to be infected, and other factors known in the art. In some embodiments, the microorganism can be a virus, including the viruses disclosed

herein. Thus, the nucleotide sequences can be contained in a recombinant virus containing appropriate expression cassettes. Suitable viruses for use herein, include, but are not limited to, baculovirus, vaccinia, Sindbis virus, Sendai virus, adenovirus, an AAV virus or a parvovirus, such as MVM or H-1. The vector can also be a retrovirus, such as MoMULV, MoMuLV, HaMuSV, MuMTV, RSV or GaLV. For expression in mammalian cells, a suitable promoter is, for example, human cytomegalovirus immediate early promoter (pCMV). Furthermore, tissue and/or organ specific promoters can be used. For example, the nucleotide sequences can be operatively linked with a promoter allowing high expression. Such promoters can include, for example, inducible promoters; a variety of such promoters are known to persons skilled in the art.

For generating protein or RNA-encoding nucleotide sequences and for constructing expression vectors or viruses that contain the nucleotide sequences, it is possible to use general methods known in the art. These methods include, for example, in vitro recombination techniques, synthetic methods and in vivo recombination methods as known in the art, and exemplified in Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Methods of transfecting cells, of phenotypically selecting transfectants cells, of phenotypically selecting transfectants and of expressing the nucleotide sequences by using vectors containing protein or RNA-encoding DNA are known in the art.

In some embodiments, the protein or RNA to be produced in the tumor can be linked to an inducible promoter, such as a promoter that can be induced by a substance endogenous to the subject, or by a substance that can be administered to a subject. Accordingly, provided herein are methods of producing a protein or RNA in a tumor, where the production can be induced by administration of a substance to a subject, and, optionally, harvesting the tumor and isolating the protein or RNA from the tumor. Such induction methods can be coupled with methods of monitoring a microorganism in a subject. For example, a microorganism can be monitored by detecting a detectable protein. In methods that include monitoring,

detection of a desired localization and/or level of microorganism in the subject can be coordinated with induction of microorganismal gene expression. For example, when a microorganismally expressed detectable protein is detected in tumor, but not appreciably in normal organs or tissues, an inducer can be administered to the subject. In another example, when a microorganismally expressed detectable protein is detected in tumor, and also in normal organs or tissues, administration of an inducer can be suspended or postponed until the detectable protein is no longer detected in normal organs or tissues. In another example, when a microorganismally expressed detectable protein is detected at sufficient levels in tumor, an inducer can be administered to the subject. In another example, when a microorganismally expressed detectable protein is not detected at sufficient levels in tumor administration of an inducer can be suspended or postponed until the detectable protein is detected at sufficient levels in the tumor.

Also provided herein are methods of producing a protein or RNA in a tumor, by administering a microorganism encoding the protein or RNA, and a suppressor of gene expression. The suppressor of gene expression can be administered for a pre-defined period of time, or until the microorganism accumulated in tumor but not in normal organs or tissues, or until sufficient levels of the microorganism have accumulated in the tumor, at which point administration of the suppressor can be terminated or suspended, which can result in expression of the protein or RNA. As will be recognized by one skilled in the art, methods similar to those provided herein in regard to monitoring a detectable protein and administering an inducer, can also apply for terminating or suspending administration of a suppressor.

In one embodiment, the microorganism is a bacterium, for example, an attenuated bacterium, such as those provided herein. Exemplary bacteria include attenuated Salmonella thyphimurium, attenuated Vibrio cholerae, attenuated Listeria monocytogenes or E. coli. Alternatively, viruses such as vaccinia virus, AAV, a retrovirus can be used in the methods provided herein. In exemplary methods, the virus is vaccinia virus. Other cells that can be used in the present methods include

mammalian cells, such as fibroma cells, including human cells such as human fibroma cells.

Any of a variety of animals, including laboratory or livestock animals can be used, including for example, mice, rats and other rodents, rabbits, guinea pigs, pigs, sheep, goats, cows and horses. Exemplary animals are mice. The tumor can be generated by implanting tumor cells into the animal. Generally, for the production of a desired polypeptide, RNA, or compound, any solid tumor type can be used, such as a fast growing tumor type. Exemplary fast growing tumor types include C6 rat glioma and HCT116 human colon carcinoma. Generally, for the production of a desired antibody, a relatively slow growing tumor type can be used. Exemplary slow growing tumor types include HT1080 human fibrosarcoma and GI-101A human breast carcinoma. For T-independent antibody production, nu-/nu- mice bearing allogenic tumor or xenografts can be used; while for T-dependent antibody production, immunocompetent mice with syngenic tumors can be used. In some embodiments, such as where the compound to be produced is a protein, the microorganism selected can be a microorganism that uses the translational components (*e.g.*, proteins, vesicles, substrates) of the tumor cells, such as, for example, a virus that uses the translational components of a tumor cell. In such instances, the tumor cell type can be selected according to the desired post-translational processing to be performed on the protein, including proteolysis, glycosylation, lipidylation, disulfide formation, and any refolding or multimer assembly that can require cellular components for completing. In some examples, the tumor cell type selected can be the same species as the protein to be expressed, thus resulting in species-specific post-translational processing of the protein; an exemplary tumor cell type-expressed protein species is human.

1. Production of Recombinant Proteins and RNA molecules

The tumor tissue can be surgically removed from the animal. After homogenization of the tumor tissue, the desired polypeptide, RNA or other biological compound can be purified according to established methods. For example, in the case of a recombinant polypeptide, the polypeptide might contain a

bindable tag such as a his-tag, and can be purified, for example, via column chromatography. The time necessary for accumulation of sufficient amounts of the polypeptide or RNA in the tumor of the animal depends on many factors, for example, the kind of animal or the kind of tumor, and can be determined by the skilled person by routine experimentation. In general, expression of the desired polypeptide can be detected two days after virus injection. The expression peaks approximately two weeks after injection, and lasts up to two months. In some embodiments, the amount of desired polypeptide or RNA in the tumor can be determined by monitoring a microorganismally expressed detectable substance, where the concentration of the detectable substance can reflect the amount of desired polypeptide or RNA in the tumor.

In another embodiment, the desired polypeptide, RNA or other compound can be manufactured in the subject, and provide a beneficial effect to the subject. In one example, a microorganism can encode a protein or RNA, or a protein that manufactures a compound that is not manufactured by the subject. In one example, a microorganism can encode a peptide hormone or cytokine, such as insulin, which can be released into the vasculature of a subject lacking the ability to produce insulin or requiring increased insulin concentrations in the vasculature. In another example, blood clotting factors can be manufactured in a subject with blood clotting deficiency, such as a hemophiliac. In some embodiments, the protein or RNA to be produced in the tumor can be linked to an inducible promoter, such as a promoter that can be induced by increased glucose concentrations. In such instances, the manufacture of the protein or RNA can be controlled in response to one or more substances in the subject or by one or more substances that can be administered to a subject, such as a compound that can induce transcription, for example, RU486. Thus, in some embodiments, the methods provided herein can include administering to a subject having a tumor, a microorganism that can express one or more genes encoding a beneficial gene product or a gene product that can manufacture a beneficial compound.

2. Production of Antibodies

Also provided are methods for producing a desired antibody, the method comprising the following steps: (a) administering a microorganism containing a nucleotide sequence encoding an antigen into an animal bearing a tumor; and (b) isolating the antibody directed to the antigen from the serum obtained from the animal. The antibodies directed to the antigen can be isolated and purified according to well known methods. Antibodies that are directed against specific contaminating antigens (*e.g.*, bacteria antigens) can be removed by adsorption, and the antibodies directed against the target antigen can be separated from contaminating antibodies by affinity purification, for example, by immuno affinity chromatography using the recombinant antigen as the ligand of the column, by methods known in the art. Antibodies can be collected from the animal in a single harvest, or can be collected over time by collection bleeds, as is known in the art.

F. Pharmaceutical Compositions, combinations and kits

Provided herein are pharmaceutical compositions, combinations and kits containing a microorganism provided herein and one or more components. Pharmaceutical compositions can include a microorganism and a pharmaceutical carrier. Combinations can include two or more microorganisms, a microorganism and a detectable compound, a microorganism and a microorganism expression modulating compound, a microorganism and a therapeutic compound. Kits can include the pharmaceutical compositions and/or combinations provided herein, and one or more components such as instructions for use, a device for detecting a microorganism in a subject, a device for administering a compound to a subject, and a device for administering a compound to a subject.

1. Pharmaceutical Compositions

Also provided herein are pharmaceutical compositions containing a modified microorganism and a suitable pharmaceutical carrier. Examples of suitable pharmaceutical carriers are known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions, etc. Such carriers can be formulated by conventional

methods and can be administered to the subject at a suitable dose. Colloidal dispersion systems that can be used for delivery of microorganisms include macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions (mixed), micelles, liposomes and lipoplexes. An exemplary colloidal system is a liposome. Organ-specific or cell-specific liposomes can be used in order to achieve delivery only to the desired tissue. The targeting of liposomes can be carried out by the person skilled in the art by applying commonly known methods. This targeting includes passive targeting (utilizing the natural tendency of the liposomes to distribute to cells of the RES in organs which contain sinusoidal capillaries) or active targeting (for example by coupling the liposome to a specific ligand, for example, an antibody, a receptor, sugar, glycolipid, protein etc., by well known methods). In the present methods, monoclonal antibodies can be used to target liposomes to specific tissues, for example, tumor tissue, via specific cell-surface ligands.

2. Host Cells

Also provided herein are host cells that contain a microorganism provided herein such as a modified vaccinia virus. These host cells can include any of a variety of mammalian, avian and insect cells and tissues that are susceptible to microorganism, such as vaccinia virus, infection, including chicken embryo, rabbit, hamster and monkey kidney cells, for example, CV-1, BSC40, Vero, BSC40 and BSC-1, and human HeLa cells. Methods of transforming these host cells, of phenotypically selecting transformants etc., are known in the art.

3. Combinations

Combinations can include a microorganism and one or more components. Any combination herein also can, in place of a microorganism, contain a pharmaceutical composition and/or a host cell containing a microorganism and one or more components.

Exemplary combinations can contain two or more microorganisms, a microorganism and a detectable compound, a microorganism and a microorganism expression modulating compound, or a microorganism and a therapeutic compound.

Combinations that contain two or more microorganisms can contain, for example, two or more microorganisms that can both be administered to a subject in performing the methods provided herein, including sequentially administering the tow microorganisms. In one example, a combination can contain a virus and a bacterium, where, for example, the virus can first be administered to the subject, and the bacterium can be subsequently administered to the subject.

Combinations provided herein can contain a microorganism and a detectable compound. A detectable compound can include a ligand or substrate or other compound that can interact with and/or bind specifically to a microorganismally expressed protein or RNA molecule, and can provide a detectable signal, such as a signal detectable by tomographic, spectroscopic or magnetic resonance techniques. Exemplary detectable compounds can be, or can contain, an imaging agent such as a magnetic resonance, ultrasound or tomographic imaging agent, including a radionuclide. The detectable compound can include any of a variety of compounds as provided elsewhere herein or are otherwise known in the art. Typically, the detectable compound included with a microorganism in the combinations provided herein will be a compound that is a substrate, a ligand, or can otherwise specifically interact with, a protein or RNA encoded by the microorganism; in some examples, the protein or RNA is an exogenous protein or RNA. Exemplary microorganisms/detectable compounds include a microorganism encoding luciferase/luciferin, β-galactosidase/(4,7,10-tri(acetic acid)-l-(2-β-galactopyranosylethoxy)-l,4,7,10-tetraazacyclododecane) gadolinium (Egad), and other combinations known in the art.

Combinations provided herein can contain a microorganism and a microorganism gene expression modulating compound. Compounds that modulate gene expression are known in the art, and include, but are not limited to, transcriptional activators, inducers, transcriptional suppressors, RNA polymerase inhibitors, and RNA binding compounds such as siRNA or ribozymes. Any of a variety of gene expression modulating compounds known in the art can be included in the combinations provided herein. Typically, the gene expression modulating

compound included with a microorganism in the combinations provided herein will be a compound that is a can bind, inhibit, or react with one or more compounds active in gene expression such as a transcription factor or RNA, of the microorganism of the combination. An exemplary microorganism/expression modulator can be a microorganism encoding a chimeric transcription factor complex having a mutant human progesterone receptor fused to a yeast GAL4 DNA-binding domain an activation domain of the herpes simplex virus protein VP16 and also containing a synthetic promoter containing a series of GAL4 recognition sequences upstream of the adenovirus major late E1B TATA box, where the compound can be RU486 (see, *e.g.*, Yu *et al.*, Mol Genet Genomics 2002 268:169-178). A variety of other microorganism/expression modulator combinations known in the art also can be included in the combinations provided herein.

Combinations provided herein can contain a microorganism and a therapeutic compound. Therapuetic compounds can include compounds that are substrates for microorganismally expressed enzymes, compound that can kill or inhibit microorganism growth or toxicity, or other therapeutic compounds provided herein or known in the art to act in concert with a microorganism. Typically, the therapeutic compound included with a microorganism in the combinations provided herein will be a compound that can act in concert with a microorganism, such as a substrate of an enzyme encoded by the microorganism, or an antimicroorganismal agent known to be effective against the microorganism of the combination. Exemplary microorganism/therapeutic compound combinations can include a microorganism encoding Herpes simplex virus thymidine kinase/gancyclovir, and streptococcus pyogenes/penicillin. Any of a variety of known combinations provided herein or otherwise known in the art can be included in the combinations provided herein.

4. Kits

Kits are packaged in combinations that optionally include other reagents or devices, or instructions for use. Any kit provided herein also can, in place of a

microorganism, contain a pharmaceutical composition, a host cell containing a microorganism, and/or a combination, and one or more components.

Exemplary kits can include the microorganisms provided herein, and can optionally include one or more components such as instructions for use, a device for detecting a microorganism in a subject, a device for administering a compound to a subject, and a device for administering a compound to a subject.

In one example, a kit can contain instructions. Instructions typically include a tangible expression describing the microorganism and, optionally, other components included in the kit, and methods for administration, including methods for determining the proper state of the subject, the proper dosage amount, and the proper administration method, for administering the microorganism. Instructions can also include guidance for monitoring the subject over the duration of the treatment time.

In another example, a kit can contain a device for detecting a microorganism in a subject. Devices for detecting a microorganism in a subject can include a low light imaging device for detecting light, for example emitted from luciferase, or fluoresced from green fluorescence protein, a magnetic resonance measuring device such as an MRI or NMR device, a tomographic scanner, such as a PET, CT, CAT, SPECT or other related scanner, an ultrasound device, or other device that can be used to detect a protein expressed by the microorganism within the subject. Typically, the device of the kit will be able to detect one or more proteins expressed by the microorganism of the kit. Any of a variety of kits containing microorganisms and detection devices can be included in the kits provided herein, for example, a microorganism expressing luciferase and a low light imager, or a microorganism expressing green fluorescence protein and a low light imager.

Kits provided herein also can include a device for administering a microorganism to a subject. Any of a variety of devices known in the art for administering medications or vaccines can be included in the kits provided herein. Exemplary devices include a hypodermic needle, an intravenous needle, a catheter, a needle-less injection device, an inhaler, and a liquid dispenser such as an

eyedropper. Typically, the device for administering a microorganism of the kit will be compatible with the microorganism of the kit; for example, a needle-less injection device such as a high pressure injection device can be included in kits with microorganisms not damaged by high pressure injection, but is typically not included in kits with microorganisms damaged by high pressure injection.

Kits provided herein also can include a device for administering a compound to a subject. Any of a variety of devices known in the art for administering medications to a subject can be included in the kits provided herein. Exemplary devices include a hypodermic needle, an intravenous needle, a catheter, a needle-less injection an inhaler, and a liquid dispenser. Typically the device for administering the compound of the kit will be compatible with the desired method of administration of the compound. For example, a compound to be delivered subcutaneously can be included in a kit with a hypodermic needle and syringe.

F. Examples

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1

Generation of recombinant viruses

A Wild type vaccinia virus (VV) strain LIVP (the well known viral strain, originally derived by attenuation of the strain Lister from the ATCC under Accession Number VR-1549, from the Lister Institute of Viral Preparations, Moscow, Russia; see, Al'tshtein *et al.*, (1983) Dokl. Akad. Nauk USSR *285*:696-699) designed as VGL was used as a parental virus for the construction of recombinant viruses designated RVGLX herein. All vaccinia viruses were purified using sucrose gradient (Yoklik). VVs were propagated and titers were determined by plaque assays using CV-1 cells (ATCC No. CCL-70). Methods for constructing recombinant vaccinia viruses are known to those of skill in the art (see, *e.g.*, Chakrabarti *et al.*, (1985 Mol. Cell Biol. *5*:3403 and U.S. Patent No.4,722,848. Table 1 summarizes the recombinant VV strains described in this Example.

Inactivation of VV by PUV treatment

LIVP VV (3 x 108 pfu/ml) was incubated with 1 μg/ml psoralen (Calbiochem, La Jolla, CA), suspended in Hank's buffer at room temperature for 10 min, and then irradiated for 5 min in Stratalinker 1800 UV crosslinking unit (Stratagent, La Jolla CA) eaquipped with five 365 nm long wave UV bulb to produce PUV-VV.

RVGL8: LacZ insertion into F3 of LIVP

Construction of recombinant vaccinia virus RVGL8 containing a lacZ gene inserted the NotI site was prepared as described in Timiryasova *et al.* (2001), BioTechniques 31, 534-540. Briefly it was prepared as follows. The BamHI/SmaI fragment (3293 bp) of pSC65 (see, Chakrabarti *et al.* (1997), BioTechniques 23, 1094-1097; see, also Current Protocols in Molecular Biology, Green Publishing and Wiley-Interscience Supplement 15:16.17.2 (1992); see also SEQ ID No. 5 herein and SEQ ID No. 57 in PCT International application No. WO 99/32646) containing the lacZ gene under the control of the vaccinia p7.5 promoter and strong synthetic vaccinia pE/L promoter was isolated by digestion with restriction enzymes, blunted with Klenow enzyme, and cloned into SmaI site of pNT8 plasmid (Timiryasova *et al.* (2001), BioTechniques *31*: 534-540) to produce pNZ2 a shuttle plasmid.

To construct pNT8, the *NotI* region of the wild type VV strain LIVP was amplified using the following primers:
Forward: 5'-GGGAATTCTTATACATCCTGTTCTATC – 3' (SEQ ID No. 3);
Reverse: 5'-CCAAGCTTATGAGGAGTATTGCGGGGCTAC-3' (SED ID No. 4)
with the VV as a template. The resulting 972 bp fragment contained flanking EcoRI and HindIII sites at the 5' and 3' ends, respectively. The PCR product was cleaved with EcoRI and HindIII and inserted in pUC28 (Benes *et al.*, (1993) Gene 130: 151. Plasmid pUC28 is prepared from pUC18 (available from the ATCC under Accession Number 37253 by introducing a synthetic oligo adaptor using primers:
pUC28 I: 5'AATTCAGATCTCCATGGATCGATGAGCT 3' (SEQ ID NO.6);
pUC28 II: 3'GTCTAGAGGTACCTAGCTAC 5' (SEQ ID No. 7) into the EcoRI and SstI sites of pUC18. This introduces BglII, ClaI, and NcoI sites into the polylinker of pUC18.

Plasmid pNZ2 contains cDNA encoding the *E. coli* lacZ gene under the control of the vaccinia virus early/late promoter p7.5 and a synthetic early/late vaccinia pE/L promoter derived from the plasmid pSC65 (see, Chakrabarti *et al.* (1997), BioTechniques 23, 1094 1097; see, also Current Protocols in Molecular Biology, Green Publishing and Wiley-Interscience Supplement 15:16.17.2 (1992); see also SEQ ID No. 5 herein and SEQ ID No. 57 in PCT International application No. WO 99/32646). Plasmid pNZ2 provides for homologous recombination of lacZ into the NotI site of the VGL virus (ATCC VR-1549), to produce in the recombinant vaccinia virus designated RVGL8. The complex of wild type vaccinia virus DNA digested with NotI and not digested plasmid DNA pNZ2 was transfected for in vivo recombination into PUV VV infected cells to produce RVGL8 (see Figure 1A and Figure 1B). RVGL8 and the other recombinant vaccinia viruses described herein are listed in Table 1, below.

Mutant Virus Formation/Transfection

CV-1 African green monkey kidney fibroblasts (ATCC No. CCL-70) grown on 60 mm dishes (Corning, Corning, NY, USA) were infected with PUV-VV (strain LIVP treated with psoralen and UV; see, *e.g.,* Tsung *et al.*. (1996), J. Virol. 70, 165-171; Timiryasova *et al.* (2001), BioTechniques 31, 534-540; Timiryasova *et al.* (2001), J. Gene 3 Med. 3, 468-477) at multiplicity of infection (MOI) of 1.

Two hours post-infection, the cells were transfected with a mixture of NotI-digested viral DNA (4 μg) and intact plasmid DNA (4 μg). Lipid-mediated transfection of cells was carried out using 5 μl of GenePORTER reagent (Gene Therapy Systems, San Diego, CA, USA) per μg of the DNA according to manufacturers' instructions. Cells were incubated in transfection mixture for 4 h and then supplemented with a medium containing 20% of fetal bovine serum. Cytopathic effects were monitored daily by light microscopy. Cells were incubated for 5-7 days until formation of the virus plaques and complete cytopathic effect. Then, infected cells were harvested, resuspended in 0.5 ml of medium, and frozen and thawed three times to release the virus. Single virus plaques were selected for

the preparation of small and large recombinant virus stocks and analyzed for the insertion and expression of the genes.

Confirm Mutant

Viral DNA was analyzed by Southern blots. Briefly, to isolate viral DNA, confluent monolayers of CV-1 cells, grown on 10 cm plates, were infected with the wild type VV (strain LIVP) or VV of the virus stock obtained from a single recombinant plaque. When the cytopathic effect was complete, cells were harvested and the pellet was resuspended in 3 ml of 10 mM Tris-HC1, pH 9.0. Viral particles were lysed, treated with proteinase K, and the virus DNA was isolated by phenol/chloroform extraction, followed by ethanol precipitation. The DNA was resuspended in 100 µl of sterile water. The viral DNA samples were digested by NotI overnight at 37°C, followed by phenol-chloroform treatment, precipitated and 10 µg of DNA samples were separated through a 0.8% agarose gel. The DNA was transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) and fixed to the membrane using a GS Gene Linker (Bio-Rad Laboratories, Hercules, CA, USA). The DIG-labeling of DNA was performed using a nonradioactive DNA labeling and detection kit (Roche Diagnostics Corporation) and incubating for 60 min at 37°C. The membrane was hybridized with a denatured DIG-labeled 3357 bp NotI-NotI DNA fragment of the plasmid pNZ2 encoding the lacZ gene. Hybridization conditions and blot development were performed as suggested by the manufacturer.

The predicted size of the band is 3357 bp. The hybridization of NotI digested viral DNAs with a 3357 bp DNA probe confirmed the integration of the lacZ gene into NotI site of virus genome.

Construction of RVGL2 and RVGL23 viruses with a single *TK* gene mutation

Vaccinia virus LIVP was used for the construction of recombinant virus RVGL2. Vaccinia virus Western Reserve (WR) was used for the construction of recombinant virus RVGL23. The cDNA of *Renilla* luciferase and *Aequorea* GFP fusion (*ruc-gfp*; 1788 bp; see, Wang *et al.*, (1996) Bioluminescence Chemiluminescence 9:419-422; Wang *et al.*, (2002) Mol. Genet. Genomics 268:160-

168; Wang *et al.* (1997) pp 419-422 in Bioluminescence and Chemiluminescence: molecular reporting with photons, Hastings *et al.*,, eds., Wiley, Chicheser UK ; see, also U.S. Patent No. 5,976,796; see also SEQ ID No. 8 herein, which sets forth a sequence for a *ruc-gfp* construct) was excised from plasmid pcDNA-*ruc-gfp* (RG), which is described in Wang *et al.*, (1996) Bioluminescence Chemiluminescence 9:419-422 and Wang *et al.*, (2002) Mol. Genet. Genomics 268:160-168 and briefly below, by restriction endonuclease PmeI and inserted into the SmaI site of pSC65 plasmid (see SEQ ID No. 5; see, also herein and SEQ ID No. 57 in PCT International application No. WO 99/32646), resulting in pSC65-RG-1 plasmid DNA.

Briefly to prepare pcDNA-*ruc-gfp*, the *Eco*RI-*Not*I fragment encoding the modified *Renilla* luciferase-ending DNA (see, Wang *et al.* (1997) pp 419-422 in Bioluminescence and Chemiluminescence: molecular reporting with photons, Hastings *et al.*,, eds., Wiley, Chicheser UK) was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA), placing expression of the *Renilla* luciferase under control of the CMV promoter. The stop codon at the end of the *Renilla* luciferase ORF was removed, and the resulting plasmid digested with *Not*I. The *Not*I fragment containing the ORF encoding humanized *Aequorea* GFP (Zolotukhin *et al.*, (1996) J. Virol. 70:4646-4654) was excised from the pTR-β-actin plasmid and inserted into the *Not*I site of the plasmid encoding the *Renilla* luciferase. The resulting plasmid was designated pcDNA-ruc- the *ruc-gfp*.

New plasmid pSC65-RG-1 containing *ruc-gfp* fusion under the control of the vaccinia PE/L promoter and E. *coli* β-galactosidase under control of p7.5 promoter of VV was used for the construction of a single *TK* gene interrupted virus RVGL2 of strain LIVP and RVGL23 of strain WR. CV-1 cells were infected with wt LIVP or wt WR virus at MOI of 0.1, and two hours later, pSC65-RG-1 plasmid DNA was transfected using FuGene6 transfection reagent (Roche). After 24 h of incubation, cells were three times frozen and thawed to release the virus. Recombinant viruses were screened on CV-cells in the presence of substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Stratagene, Cedar Creek, TX, USA). After four

cycles of virus purification, all virus plaques were positive for β-galactosidase expression. The expression of the ruc-gfp fusion protein was confirmed by luminescence assay and fluorescence microscopy, respectively. Schematic maps of the viruses are set forth in Figure 1B

Construction of RVGL5 and RVGL9 viruses with single gene mutations

Recombinant vaccinia virus RVGL5 contains the lacZ gene under the control of the vaccinia late p11 promoter inserted int the HA gene of vaccinia genome (Timiryasova *et al.* (1993) Mol Biol 27:392-402; see, also, Timiryasova *et al.*, (1992) Oncol. Res *11*:133-144.). Recombinant vaccinia virus RVGL9 contains a fusion of the Renilla luciferase gene (ruc) and cDNA of green fluorescence protein (gfp) under the control of a synthetic early/late vaccinia promoter (PE/L) inserted into the F3 gene of the VV genome (Timiryasova *et al.*, (2000)) pp. 457-459 in Proceedings of the 11th International Symposium on Bioluminescence and Chemiluminescence, Case et al., eds). RVGP5 and RVGLP9 were constructed as described for RVGLP2 and RVGLP23.

Construction of RVGL20 virus with double *TK* and F3 gene mutations

The cDNA of human trasferrin receptor *(hTR)* (2800 bp) with polyA sequence was isolated from pCDTR1 plasmid (ATCC Accession No. 59324 and 59325) by *Bam*HI, treated with *Klenow* and inserted into *Sal*I site of pSC65 plasmid (SEQ ID No. 5 herein and SEQ ID No. 57 in PCT International application No. WO 99/32646), resulting in pSC-TfR and pSC-rTfR. Plasmid pSC-rTfR contains cDNA hTR in an orientation opposite to the vaccinia PE/L promoter and E.coli β-galactosidase under control of the early/late vaccinia p7.5 promoter flanked by vaccinia sequences for insertion into vaccinia *TK* gene. pSC-rTfR was used for the construction of RVGL20 virus. RVGL9, a recombinant virus with single deletion carrying ruc-gfp fusion in the F3 gene locus, which contains a unique *Not*I site in the LIVP strain (see above, see, also, Timiryasova *et al.*, (2000) pp. 457-459 in *Proceedings of the 11th International Symposium on Bioluminescence and Chemiluminescence*, Case *et al.*, eds) , was used as a parental virus for the creation

of RVGL20 virus by homologous recombination as described above. A schematic of RVGL20 virus is set forth in Figure 1B

Construction of RVGL21 virus with triple *TK*, *F3* and *HA* gene mutations

The cDNA of the β-glucuronidase (gus)of E. *coli* (1879 bp) was released from pLacGus plasmid (Invitrogen; see SEQ ID No. 9 herein) with *Xba*I (blunt ended with Klenow fragment) and *Hin*dIII, and cloned into pSC11 plasmid pSC65 (Chakrabarti *et al.*(1985) Mol. Cell Biol. *5*:3403-3409; SEQ ID 5 herein and SEQ ID No. 57 in PCT International application No. WO 99/32646) digested with *Xho*I (treated with Klenow) and *Hin*dIII under the control of a vaccinia p11 late promoter, resulting in a plasmid pSC-GUS. The *Sma*I-*Hin*dIII fragment from pSC-GUS plasmid was inserted into pVY6 plasmid , a vector for inserting antigen genes into the hemagglutinin gene of vaccinia (see, *e.g.*, Flexner *et al.*, (1988) Nature *355*:259-262; Flexner *et al.*, (1988) Virology *166*: 339-349; see also U.S. Patent No. 5,718,902) digested with *Sma*I and *Bam*HI, resulting in pVY-GUS plasmid. The resulting plasmid, designated pVY-GUS plasmid, contains the cDNA encoding *gus* under the control of the vaccinia late promoter p11 flanked by vaccinia sequences for insertion into the hemagglutinin *(HA)* gene. Recombinant virus RVGL20 with double deletions was used as the parental virus for the construction of RVGL21 virus. CV-1 cells were infected with RVGL20 virus at MOI of 0.1. Two hours after infection, cells were transected with pVY-GUS plasmid DNA using FuGene6 transfection reagent (Roche). Recombinant virus plagues were selected in CV-1 cells by color screening upon addition of β-glucuronidase substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronicacid (X-GlcA) (Research Products Int. Co., Mt. Prospect, IL, USA) into agar medium. After eight cycles of purification in agar medium in the presence of X-GlcA pure recombinant virus RVGL21 was selected. RVGL21 virus has interruptions of *TK*, *F3* and *HA* genes and is presented schematically in Figure 1B.

***In vitro* virus growth**

CV-1, C6 (ATCC No. CCL-107), B16-F10 (ATCC No. CRL-6475), and GI-101A (Rumbaugh-Goodwin Institute for Cancer Research Inc. Plantation, FL; U.S. Pat. No. 5,693,533) cells were seeded in 24-well plates at the density of 1 x 10^5, 2 x 10^5, 4 x 10^5, and 2 x 10^5 cells/well, respectively. The next day, the cells were simultaneously infected with 0.001 or 0.01 PFU/cell of a wild type LIVP and its mutants. The virus suspension was added to cell monolayer (0.15 ml/well) and incubated at 37°C for 1 h with brief agitation every 10 min. Then, the virus was removed, appropriate complete growth medium was added (1 ml/well), and the cells were then incubated at 37°C for 24, 48, 72 and 96 h after virus infection. To establish resting cell culture, a confluent monolayer of CV-1 cells was incubated for 6 days in DMEM with 5 % FBS at 37°C. These resting cells were infected and harvested at the same time points after infection as described above. Virus from the infected cells was released by one cycle of freezing and thawing. Viral titers were determined in duplicates by plaque assay on CV-1 cells and expressed as PFU/ml.

Table1

List of recombinant vaccinia viruses (VV)

Designation	Prior Designation	Description	InsertionLocus/loci	Reference
VGL	wt VV	strain LIVP VV	No Insertions	Publicly available
RVGL1	recVV2	(p7.5) Luc-(p11) LacZ of LIVP VV	HindIII-N-Interrupted	Timiryasova TM, Kopylova-Sviridova TN, Fodor I. Mol. Biol. (Russian) 27:392-401 (1993); Timiryasova TM, Li J, Chen B. Chong D. Langridge WHR, Gridley DS, Fodor I. Oncol. Res. 11:133-144 (1999)
RVGL5	recVV8	(p11) LacZ of LIVP VV	HA-Interrupted	Timiryasova TM, Kopylova-Sviridova TN, Fodor I. Mol. Biol. (Russian) 27:392-401 (1993)
RVGL7	rVV-EGFP or rVV-GFP	(PE/L) EGFP-(p7.5) LacZ of LIVP VV	TK-Interrupted	Umphress S, Timiryasova T., Arakawa T, Hilliker S, Fodor I, Langridge W. Transgenics 4:19-33 (2003)
RVGL8	rVV-Not-LacZ or rVV-Not-LZ	(p7.5) LacZ of LIVP VV	NotI (F3)-Interrupted	Timiryasova TM, Chen B, Fodor N, Fodor I. BioTechniques 31:534-540 (2001)
RVGL9	rVV-RG or rVV-ruc-gfp	(PE/L) Ruc-GFP of LIVP VV	NotI (F3)-Interrupted	Timiryasova TM, Yu Ya, Shabahang S, Fodor I, Szalay AA. Proceedings of the 11th International Symposium on Bioluminescence & Chemiluminescence pp.457-460 (2000)
RVGL12		Same as RVGL7, except that HSV TK is inserted in place of gfp		
RVGL19		(PE/L) Trf-(p7.5) LacZ in Tk locus (PE/L) Ruc-GFP in	TK- and NotI (F3)-Interrupted	Herein

		F3 locus of LIVP VV		
RVGL20		(PE/L) rTrf-(p7.5) LacZ in TK locus (PE/L) Ruc-GFP in F3 locus of LIVP V	Tk- and NotI (F3)- Interrupted	Herein
RVGL21		(PE/L) rTrf-(p7.5) LacZ in TK locus, (p11) LacZ in HA locus, (PE/L) Ruc-GFP in F3 locus of LIVP VV	Tk-, HA-interrupted and NotI (F3)- Interrupted	Herein
RVGL23		(PE/L) rTrf-(p7.5) LacZ in TK locus of WR VV	Tk- Interrupted	Herein

Example 2

In vitro analysis of virus levels

LacZ

Analysis of *lacZ* expression induced by recombinant vaccinia virus was performed as described previously (Timiryasova *et al.* (2001), BioTechniques 31, 534-540). Briefly, CV-1 cells grown 6-well plates (Corning, Corning, NY, USA) were infected with ten-fold dilutions of the virus stock. The virus was allowed to absorb for 1 h at 37°C with occasional rocking. Then, the virus inoculum was replaced with a complete medium containing 1% of agar, and the incubation was carried out for 48 h. To visualize the virus plaques, 300 µg of X-Gal (Molecular Probes, Eugene, Oregon, USA) per ml and 0.1% of neutral red (Sigma, St. Louis, MO, USA) were added to the second agar overlay, and plaques were counted and isolated after 12 h incubation at 37°C. Levels of vaccinia virus in cells in vitro could also be determined by measuring the plaque forming units (PFU) in the cells.

***In vitro* infectivity of VV's measured by Plaque Forming Units**

The ability of wt LIVP virus and its mutants to infect and replicate was analyzed in dividing and resting CV-1 cells as well as in three tumor cell lines (C6, GI-101A, B16-F10). The results demonstrate that vaccinia mutants can efficiently infect and replicate in dividing CV-1 cells at an MOI of 0.001. A significant yield of vaccinia virus was obtained from dividing CV-1 cells. The yield of wt VV and its mutants in dividing CV-1 cells was about 10 times higher than in resting CV-1 cells. There was no significant difference in viral recovery between vaccinia mutants and wt virus *in vitro* studies. The interruption of *TK, F3* and *HA* genes made no difference to VV mutants replication in the dividing CV-1 cells. Three tumor cells were tested. The relative sensitivities to cytopathic effects at MOI of 0.001 were follows: CV-1 (dividing, highest), CV-1 (resting), C6, GI-101A, B16-F10 (lowest). Mouse B16-F10 melanoma cells were not sensitive to virus infection at MOI of 0.001. Very low viral titer was recovered from melanoma cells infected at MOI of 0.01. Also observed was that wt WR strain was able to infect melanoma cells *in vitro* more efficiently compared to LIVP strain and virus recovery was higher compared to LIVP strain.

Example 3

Animal models and assays

Animal Models

Athymic nude mice (*nu/nu*) and C57BL/6 mice (Harlan Animal Res., Inc., Wilmington, MA) at 6-8 weeks of age were used for animal studies. Mice in groups of five or four were infected i.v. with 10^7 PFU of VV in a volume of 0.1 ml i.v. Mice were imaged by low-light imager and fluorescence imager for ruc and for gfp expression, respectively. The study was approved prior to initiation by the Animal Research Committee of LAB Research International Inc. (San Diego, CA, USA). All animal care was performed under the direction of a licensed veterinarian of LAB Research International Inc. (San Diego, CA, USA).

Glioma Model

To establish subcutaneous glioma tumor, rat glioma C6 cells (ATCC No. CCL-107) were collected by trypsinization, and 5 x 10^5 cells/0.1 ml/mouse were

injected subcutaneously (s.c.) into right hind leg of 6-8 week old male athymic mice. On day 7 after C6 cell implantation when median tumor size was about 150 mm^3, viruses at the dose of 10^7 PFU/0.1ml/mouse were injected intravenously (i.v.). Mice were sacrificed 14 days after virus injection. In the kinetic studies using of RVGL9 virus, mice were sacrificed at 20 min, 1 h, 4 h, 18 h, 36 h, 3 d, 5 d, 7 d and 14 days after virus injection.

Breast Tumor Model

To develop sub cutaneous (s.c). breast tumor, human breast cancer GI-101 A cells (Rumbaugh-Goodwin Institute for Cancer Research Inc. Plantation, FL; U.S. Pat. No. 5,693,533) at the dose of 5 x 10^6 cells/0.1 ml/mouse were injected s.c. into the right hind leg of 6-8 week old female athymic mice. On day 30 after GI-101A cell implantation, when median tumor size was about 500 mm^3, viruses at the dose of 10^7 PFU/mouse were injected i.v. Mice were sacrificed on day 14 after virus injection. Mice for survival experiments and breast tumor therapy studies were kept for long time periods (more than 100 days after virus injection). Mice that developed tumor with the size about 4000 mm^3, and/or lost 50% of body weight were sacrificed.

Melanomal Model

For a melanoma model, mouse melanoma B16-F10 cells (ATCC No. CRL-6475) at the dose of 2 x 10^5 cells/0.04 ml/mouse were injected into the foot pad of 6-8 week old male C57BL/6 mice. When the tumor was established (median size of tumor about 100 mm^3), on day 18 after cell implantation, viruses at the dose of 10^7/mouse were injected i.v. Mice were sacrificed 10 days after virus injection.

Vaccinia Virus in Animal Models

Vaccinia virus recovery from tumor and organs of nude mice

From sacrificed animals blood was collected, and organs (lung, liver, spleen, kidneys, testes, ovaries, bladder, brain, heart) and tumors were harvested and homogenized in PBS containing a mixture of protease inhibitors. Scissors and forceps were changed after each organ dissection or incision to avoid cross-contamination of the tissues. Samples were frozen and thawed, centrifuged at 1,000

g for 5 min. Viral titer was determined in the supernatant diluted in serum-free medium on CV-1 cells by plaque assay and staining them with 1% (wt/vol) crystal violet solution after 48 h incubation. Each sample was assayed in duplicate and viral titer was expressed as mean PFU/g of tissue.

Assay Measurements

Survival studies were performed on 6-week old nude mice bearing s.c.. human breast tumor. Mice were injected i.v. with 10^7 of vaccinia viruses and followed for survival. Individual body weight was measured twice a week. Gain/loss of body weight after virus infection was calculated as the percentage: body weight (g) - tumor weight (g) on day of virus injection / body weight (g) - tumor weight (g) on day of monitoring x 100%. Spleens were excised from euthanized animals and weighed. The RSW was calculated as follows: RSW =weight of spleen (g) x 10^4/animal body weight (g)- tumor weight (g). Mice were euthanized when the mean tumor volume reached 3000 mm^3 or developed the signs of disease. Rapid CO_2 euthanasia was humanely performed in compliance with the *NIH Guide for the Care and Use of Laboratory Animals.*

Reporter genes assays

LacZ

E. *coli* β-galactosidase activity in tissue samples and in the serum of the mice was determined using chemiluminescent Galacto-Light Plus™ Assay system (Applied Biosystems, Bedford, MA, USA) according to the instructions of the kit manufacturer. Briefly, l-20 µl of the sample was transferred into the tube with 200 µl of 1:100 diluted Reaction Buffer Diluent and incubated at RT for 30 min. A 300µ1 aliquot of accelerator (-II) was added into the tube with the sample, mixed quickly and the signal was read using luminometer. β-galactosidase activity was expressed as relative light units (RLU) per g of tissue. Purified E. *coli* β-galactosidase (Sigma) was used as a positive control and to generate a standard curve.

Luciferase

Renilla luciferase activity was measured in the supernatant of the tissue samples after they had been homogenized using a Turner TD 20e luminometer (Turner Designs, Sunnyvale, CA, USA) as described previously (Yu and Szalay, 2002) with some modifications. In brief, 20 µl of the samples was added into 500 µl of luciferase assay buffer (0.5 M NaCl, 1 mM EDTA, 0.1 M potassium phosphate pH 7.4) containing a substrate coelentrazine. Luciferase activity was measured during 10-s interval and expressed as RLU per g of tissue.

Assay Results

Tumor-selective replication of vaccinia virus RVGL8.

Recovery of wt LIVP (VGL) and RVGL8 from tumor samples and different organs from six mice and from normal organs. VGL was recovered from tumor, testes, bladder, and liver and as well as from brain. Recombinant virus RVGL8, however, was found mostly in tumor only (in mouse #24 virus was found in testes, bladder and liver; in mouse #22 in testes) and no virus was recovered from brain tissue in six tested animals. This finding demonstrates the safety of RVGL8 with the interruption of the NotI site ,

Presence of RVGL9 over time

A vaccinia virus RVGL9 with a single *F3* gene mutation and carrying ruc-gfp was used to assess the pattern of vector tissue distribution following i.v. administration into immunocompromised athymic mice bearing s.c. glioma tumors. The tissue distribution data using this recombinant virus showed virus distribution and tumor targeting by this VV strain. Kinetics studies were performed by noninvasive imaging of virus replication in the mice based on ruc and gfp expression. Four to five animals per group bearing s.c. rat glioma C6 tumor were injected with 10^7 of RVGL9 virus via the tail vein. The animals were sacrificed at 20 min, 1,4, 18 and 36 hours, 3, 5, and 14 days after virus injection. No viable viral particles were recovered from brain, bladder or testes at any time point after i.v. injection of virus. Some viral particles were recovered from spleen, heart and lung at early time points after virus injection. After 18 h post-infection, the titer of

RVGL9 virus in these organs decreased. No virus was recovered in the heart tissue after 18 h; around 156.5 and 44 PFU/g tissue was recovered from spleen and lung, respectively, on day 14 as compared to 3221.0 and 3521.9 PFU/g tissue at 20 min after virus injection, respectively. The pattern of virus recovery from liver and kidneys was different from the pattern in the spleen, heart, or lung. No virus in the kidneys and 174.9 PFU/g tissue of virus was recovered from liver at an early time after virus injection. On day 5 after virus injection, the titer of virus in these organs increased and went down on day 14 post virus injection. In tumor tissue virus was detected starting 18 h after virus administration (1.6×10^3 PFU/g tissue), and dramatically increased over the time of observation (1.8×10^8 PFU/g tissue on day 7). Virus in the tumor tissue was detectable for more then 60 days after a single i.v. virus injection. The results demonstrate tumor-specific replication of these vaccinia mutants. A correlation was observed between the virus recovery and the transgene expression in tumors and in organs. Based on the data of RVGL9 virus kinetics, day 10 or day 14 was used for tissue distribution studies of different vaccinia mutants in melanoma and glioma and breast tumor models, respectively.

Presence of various VV in mice bearing a glioma tumor

To examine tissue distribution of vaccinia virus in immunodeficient mice bearing an s.c. glioma tumor, viruses were injected i.v. at a dose of 1×10^7 PFU/0.1 ml/mouse on day 7 after C6 rat glioma cell implantation. Fourteen days after virus injection, mice were sacrificed and virus titer was determined in different tissues. Mice injected with wt WR virus were sick and dying due to viral pathogenicity. Hence, WR-injected mice were sacrificed on day 7 after virus injection. Wild type LIVP virus was recovered from all analyzed tissues as well as from brain. The amount of recovered virus particles from the mice injected with wt LIVP was much lower than wt WR strain of VV. The results presented in Table 1A

Table 1A. Viral recovery from nude mice tissues in glioma model.[a]

	LIVP Wt	RVGL2 TK-	RVGL5 HA-	RVGL9 F3-	RVGL20 TK-, F3-	RVGL21 TK-, F3-, HA-	WR[b] Wt	RVGL23 TK-, WR
Brain	1.2×10^3	1.4×10^3	0	0	0	0	1.4×10^7	1.9×10^6
Kidneys	6.1×10^2	6.7×10^2	1.6×10^2	34.6	33.3	36.6	5.4×10^6	7.9×10^2

	LIVP Wt	RVGL2 TK-	RVGL5 HA-	RVGL9 F3-	RVGL20 TK-, F3-	RVGL21 TK-, F3-, HA-	WR[b] Wt	RVGL23 TK-, WR
Lung	2.9×10^3	0	1.6×10^2	1.4×10^4	6.7×10^3	2.4×10^3	1.9×10^6	2.1×10^3
Spleen	1.9×10^2	0	1.8×10^2	1.0×10^3	1.0×10^2	1.7×10^2	1.6×10^6	1.8×10^3
Testes	5.8×10^4	64.3	6.4×10^2	7.5×10^2	0	0	9.8×10^4	1.7×10^3
Bladder	6.4×10^3	0	0	2.9×10^3	0	0	2.8×10^5	1.2×10^3
Liver	3.4×10^4	63.6	4.2×10^2	33.6	96.6	30.8	7.1×10^3	5.6×10^3
Heart	6.0×10^3	0	0	0	0	0	1.4×10^5	0
Serum[c]	0	0	0	0	0	0	6.0×10^2	0
Tumor	5.4×10^7	1.5×10^7	3.8×10^7	2.9×10^7	3.9×10^7	1.9×10^7	1.9×10^8	3.7×10^7

The results demonstrate that 10000-fold more virus was recovered in the brain of mice injected with WR strain versus wt LIVP strain. Wild type WR strain virus was recovered from the serum (600 PFU/20 µl) of mice on day 7 after virus injection. No virus was recovered in the serum of the mice injected with LIVP mutants on day 14. The level of wt LIVP in serum was not tested on day 7. About 1.9×10^6 PFU/g tissue of TK-mutant of WR strain (RVGL23) was found in the brain tissue compared to 1.4×10^3 PFU/g tissue for mice injected with the TK- mutant of LIVP strain (RVGL2).

All other mutants of VV strain LIVP were found mostly in tumor only and no virus was recovered from brain tissue of mice injected with a double or triple mutant (Table 1A). Three times as many virus particles were recovered from the tumorsof mice injected with WR compared to wt LIVP. The mean of viral recovery in tumor tissue of the mutants of LIVP strain was similar to the wt LIVP and equivalent to TK- mutant of WR strain.

Presence of various VV in mice bearing a breast tumor

Data for tissue distribution in immunocompromised mice bearing s.c. GI-101A human breast are presented in Table 1B:

Table 1B. Viral recovery from nude mice tissues in breast cancer model.

	LIVP Wt	RVGL2 TK-	RVGL5 HA-	RVGL9 F3-	RVGL20 TK-, F3-	RVGL21 TK-, F3-, HA-	WR[b] Wt	RVGL23 TK-, WR
Brain	0	0	0	0	0	0	7.2×10^6	1.6×10^4
Kidneys	3.6×10^3	38.3	27	3.3×10^2	25.8	0	3.2×10^7	2.8×10^5
Lung	8.6×10^3	5.5×10^2	29.1	1.6×10^3	1.6×10^3	1.0×10^3	2.1×10^6	3.7×10^3
Spleen	5.5×10^3	99.5	0	1.8×10^2	0	0	1.6×10^6	1.8×10^3
Ovaries	1.6×10^3	0	0	0	0	0	8.0×10^7	2.7×10^7

	LIVP Wt	RVGL2 TK-	RVGL5 HA-	RVGL9 F3-	RVGL20 TK-, F3-	RVGL21 TK-, F3-, HA-	WR[b] Wt	RVGL23 TK-, WR
Bladder	3.9×10^5	0	0	0	0	0	2.8×10^4	1.2×10^3
Liver	1.2×10^4	0	1.7×10^2	5.2×10^2	1.7×10^2	1.0×10^2	4.0×10^5	4.8×10^5
Heart	1.4×10^2	0	0	58.2	4.6×10^2	0	6.3×10^4	2.2×10^3
Serum[c]	0	0	0	0	0	0	2.4×10^3	0
Tumor	8.6×10^8	1.0×10^9	2.5×10^8	1.1×10^9	5.6×10^8	1.0×10^9	2.9×10^9	6.6×10^8

About 10-fold more viral particles were recovered from breast tumor tissue compared to glioma tumor tissue. No virus particles were recovered from the brain tissue of mice injected with either wt LIVP or its mutants. 7.2×10^6 and 1.6×10^4 PFU/g was recovered from brain tissue of mice injected with wt WR and TK-virus of WR strain VV, respectively (Table 1B). During the dissection of organs from euthanized mice, it was found that the ovaries from the mice being injected with wt WR and TK- of WR virus were drastically enlarged as compared to all other groups of mice. The analysis of viral recovery from ovaries demonstrated high titer of wt WR and TK- WR strain in ovaries, for example, 8.0×10^7 and 2.7×10^7 PFU/g, respectively. About 1.6×10^3 PFU/g was recovered from the ovaries of the mice injected with wt LIVP virus, however no virus particles at all were recovered from either ovaries or from brain of mice injected with the mutants derived from LIVP strain (Table 1B).

Presence of various VV in mice bearing a melanoma tumor

The tissue distribution of VV in the immunocompetent mice bearing melanoma tumors on foot pads also were studied. BL/6 mice on day 17 after B16F10 melanoma cell implantation were i.v. injected with the viruses at the dose of 10^7 PFU/mouse via the tail vein. All groups of mice were sacrificed on day 10 after virus injection due to huge tumor size in the PBS-injected control group. The results are set forth in Table 1C:

Table 1C. Viral recovery from C57BL/6 mice tissues in melanoma model.

	LIVP Wt	RVGL2 TK-	RVGL5 HA-	RVGL9 F3-	RVGL20 TK-, F3-	RVGL21 TK-, F3-, HA-	WR[b] Wt	RVGL23 TK-, WR
Tumor	5.4×10^6	3.9×10^6	3.7×10^5	9.5×10^5	2.5×10^5	2.4×10^5	9.9×10^6	2.2×10^6
Tissues[c]	0	0	0	0	0	0	0	0

[a] Mean of viral recovery PFU/g of tissue for 3-5 mice/group.
[b] Mice were sacrificed on day 7 after virus injection.
[c] PFU/20 µl of serum
[d] Mice were sacrificed on day 9 after virus injection.
[e] No virus was recovered in all tested tissue.

. No virus was recovered from kidneys, lung, spleen, brain, testes, bladder, liver, heart, and serum of the immunocompetent mice injected with the viruses. Virus was only recovered from the tumor tissue. About 10-fold virus particles were recovered from the tumors of mice injected with wt LIVP, TK- LIVP, wt WR, and TK- WR compared to other groups.

Example 5

Reduction of human breast tumor implanted in nude mice by recombinant vaccinia viruses RVGL7, RVGL9 and RVGL21

RVGL7 and RVGL9

Figure 1B shows a schematic representation of the recombinant vaccinia viruses used for these experiments. RVGL7 was prepared as described for the preparation of RVGL9. RVGL7 contains nucleic acid encoding EGFP and lacZ, and includes pE/L and p7.5 regulator regions inserted into the TK gene.

Luminescence and fluorescence images of tumors in a nude mouse

Human breast GI-101A cancer cells ($5x10^6$ cells/mouse) were subcutaneously implanted into the right thigh of the mice. Thirty days after cell implantation RVGL9, the *Not*I (F3)-interrupted virus expressing a fusion of *Renilla* luciferase and green fluorescence protein (RVGL9 = rVV-RG = rVVruc-gfp) was injected intravenously via tail vein at a dose of $1x10^7$ PFU/mouse. A fluorescence image of GFP and low-light image of luciferase expression were taken nine days after virus injection, *i.e.* 39 days post cell implantation showing dissemination of the virus .

Reduction of human breast tumor implanted into nude mice by vaccinia viruses RVGL7 or RVGL9

Human breast GI-101A cancer cells ($5 x 10^6$ cells/mouse) were subcutaneously implanted into the right thigh of the mice. Mice were injected i.v. with RVGL7=rVV-GFT=TK- or RVGL9-rVV-ruc-gfp=NotI (3) –interrupted viruses

(1×10^7 PFU/mouse in 0.1 ml) and PBS control on day 30 after cell implantation. Images were taken on day 65 after GI-101A cell implantation and 35 days after virus or PBS injection. The results demonstrate drastic reduction of tumor volume in the mice injected with TK- or NotI (F3) –interrupted vaccinia viruses compared with the tumor in the mice injected with PBS.

GFP in Human Breast Tumor after Viral Administration

Human breast GI-101A cancer cells (5×10^6 cells/mouse) were subcutaneously implanted into the right thigh of the mice. Mice were injected i.v. with RVGL7=rVV-GFP=TK- or RVGL9=rVV-RG-rVV-ruc-gfp-NotI (F3) –interrupted viruses (1×10^7 PFU/mouse in 0.1 ml) on day 30 after cell implantation. The data demonstrate GFP expression in tumor area in the mice injected with TK- or NotI (F3) –interrupted vaccinia viruses. No GFP signals were observed in other parts of the mice bodies. The results also showed that expression of GFP can be visualized as early as 48 h after virus injection through the tail vein. On day 16 after virus injection very strong signals of GFP which correspond to a tumor volume of about 1300-1620 mm^3 for TK- or NotI (F3) -interrupted virus, respectively were observed. Reduced GFP signals were observed on day 25 (1218-1277 mm^3 for TK- or NotI (F3) - interrupted virus, respectively) and 32 (514-887 mm^3 for TK- or NotI (F3) - interrupted virus, respectively) due to reduction of tumor volume.

Time course of Breast Tumor Volume over Time

G1-101A breast cancer cells were implanted subcutaneously into the right thigh of 4-5-week old female athymic (nu/nu) mice in the dose of 5×10^6 cells/mouse. Thirty days after tumor implantation, when the tumor reached about 500 mm^3 in volume, a single dose (1×10^7 PFU/mouse in 0.1 ml) of RVGL7 = rVV-GFP = TK- or RVGL9 = rVV-RG = rVV-ruc-gfp = NotI (F3) -interrupted vaccinia viruses or PBS control was injected intravenously (via tail vein). Tumor dimensions were measured with vernier caliper twice a week and volumes were calculated as (LxHxW) /2, where L, H and W represent the length, width, and height of the tumor, respectively and expressed in mm^3. The data demonstrate significant (60-80% on day 65) tumor reduction in the mice injected with TK-, NotI (F3) -interrupted

vaccinia viruses. In contrast, tumors grew very rapidly in the mice injected with PBS.

Monitoring of tumor regression by light extinction.

Subcutaneous GI-101A breast tumor reduction occurred in 100% of immunocompromised mice treated with a single i.v. injection of wt LIVP, single F3-, single TK-, and double F3-, TK-, mutants of LIVP strain. Some degree of toxicity was seen in the mice treated with above viruses. RVGL21 virus with the triple deletions TK, F3 and HA genes which showed no toxicity in nude mice; hence this virus was used for long-term studies. The difference in antitumor activity and survival between high and low doses of treatment using the triple mutant RVGL21 virus was not significant. GFP expression in tumor area in the mice injected with RVGL21 was monitored. No GFP signals were observed in other parts of mice body. Expression of GFP can be visualized as early as 48 h after virus injection through tail vein. On day 16 after virus injection we observed very strong signals of GFP, which correspond to tumor volume about 1300-1620 mm^3 and reduced GFP signals on days 25 (1218-1277 mm^3) and 32 (514-887 mm^3) due to reduction of tumor volume . Tumor volume reduction also was apparent by visual inspection of the mice.

Example 6

: Reduction of vaccinia virus toxicity and virulence

Reduction of vaccinia virus pathogenicity by monitoring mouse body weight and survival

The percentage of body weight change in athymic and immunocompetent mice bearing different s.c. tumors after i.v. administration of the viruses was examined. Injection of wt LIVP and wt WR and some mutants at the dose of 10^7 pfu/mouse via the tail vein led to a progressive vaccinia virus infection within a two week observation period. At one week after challenge, the mice showed typical blister formation on the tail and footpad. Later, weight loss, sometimes accompanied by swelling of the mouth region, in several cases led to death of the mice. In the case of wt WR strain of VV, mice started to die on day 7 after i.v.

injection of virus. While mice receiving the recombinant LIVP viruses gained weight or remained the same weight over the same time period.

Body weight in glioma model nude mice

Rat glioma C6 cells at the dose of $5x10^5$/0.1 ml/mouse were implanted s.c. into the right thigh of nude mice (5-6 old male mice) on day 0. Vaccinia viruses were injected i.v. (via tail vein) at the dose of 1 x 10^7 PFU/0.1 ml/mouse on day 7. Animals were weighed twice a week. Gain/loss of body weight on day 14 post infection was calculated as the percentage: body weight - tumor weight on day of virus injection (g) / body weight-tumor weight on day 14 (g) x 100%. Injection of VGL (wild type vaccinia virus, strain LIVP) and RVGL5 (HindIII-N-interrupted) causes toxicity in nude mice: mice continue to lose the weight. Recombinant vaccinia viruses RVGL5 (HA-interrupted), RVGL7 (TK-interrupted), RVGL8 (NotI(F3) -interrupted), RVGL19 (double, TK- and NotI (F3) -interrupted) were less toxic in nude mice: after losing some body weight, 10 days post-infection, mice started to gain the body weight .

Nude mice with glioma that were injected with wild type WR strain of VV lost 31.9% of body weight on day 7 after virus injection. Mice injected with TK- virus of WR strain lost 22.4% of body weight on day 14 after virus injection compared to 1.5% in the group of mice injected with TK- virus of LIVP strain of VV. All mice injected with wild type LIVP strain survived for at least 14 days (the duration of the experiment). Mice without tumor injected with VGL (wt VV, strain LIVP) lost 11.23 % of body weight. Mice bearing tumor injected with VGL (wt VV) or with RVGL1 (HindIII-N-interrupted) lost 15.79% and 10.18% of body weight, respectively. Mice in the wt LIVP group lost 15.8% of body weight versus 9.4% in the PBS injected group. Tumor-bearing mice injected with RVGL2 (TK-), RVGL5 (HA-), RVGL7 (TK-), RVGL8 (F3–), RVGL9 (F3-), RVGL20 (TK-, F3-), RVGL21 (TK-, F3-, HA-) on day 14 after virus injection lost only 1.5%, 0.4%, 2.1%, 5.0%, 7.3%, 2.4%, and 3.2% of body weight, respectively. Tumor-bearing mice injected with virus carrying double gene interruption, RVGL19 (TK- and F3–) demonstrated 0.73% gain of body weight compared to the body weight on day 0.

Based on the results of body weight, a single interruption of HA, TK, F3 (NotI site) and double interruption of TK, F3 (NotI site) genes in vaccinia virus genome reduces virulence and toxicity of the vaccinia virus strain LIVP.

Injection of wt VV strain WR, however, was extremely toxic to nude mice, which died on day 7 after virus injection. Wild type and mutant VVs of strain LIVP were less toxic in nude mice. Although nude mice injected with various LIVP strains lost some body weight, after day 10-post infection mice started to gain the body weight.

Body weight in breast tumor model athymic mice

The body weight change of athymic mice with s.c. GI-101A human breast tumor after i.v. injection of vaccinia viruses was monitored . Mice injected with wt WR strain lost 25.6% of body weight and died due to virus toxicity. Although mice injected with wt LIVP virus survived for longer time, mice lost 26.4% of body weight. Mice injected with TK-WR strain lost 17.8% of body weight, while mice injected with TK- LIVP virus gained 1.9% of body weight. All mice injected with other mutants of LIVP strain were stable; no virus related toxicity was observed in these mice.

Body weight in melanoma model immunocompetent mice

The toxicity of the vaccinia viruses in immunocompetent C57BL/6 mice bearing mouse B16-F10 melanoma on their foot pad was studied. Although mice in all groups survived during the experiment, wt WR strain was more toxic in immunocompetent mice compared to wt LIVP and recombinant strains. Mice injected with wt WR strain lost about 11.4% of body weight on day 10 after i.v. injection of virus, while mice injected with wt LIVP strain and its double (RVGL20) and triple (RVGL21) mutants lost only 2.2%, 1.3%, and 0.6% of body weight, respectively, versus to 7.1% of body weight lost in PBS injected mice. Mice administered i.v. with RVGL2 (TK-), RVGL5 (HA-), RVGL9 (F3-), and RVGL23 (TK-WR strain) continued to gain weight over this same period.

Long-term survival after viral infection for breast tumor-bearing mice

To examine the effect of different mutations on long-term survival, mice bearing s.c. GI-101A human breast tumor received doses of 10^7 virus i.v., and wereobserved for survival after viral infection. The results showed that there are differences in survival depending upon the virus injected. Injection of the nude mice bearing s.c. breast tumor with wt WR strain (i.v., 1 x 10^7/mouse) resulted in 100% mortality: four mice of five died on day 9 and one mouse died on day 11 after virus injection. Mice injected with strain LIVP survived for 35 days. Mice injected with a single mutated virus RVGL9 (F3-) developed the toxicity and 25% of mice died on day 34 after virus injection, however the deletion of F3 gene in LIVP strain prolonged the survival of mice up to 57 days. Mice injected with double mutant virus RVGL20 (F3-, TK-) began to die on day 34 after virus injection, but survived longer than F3- injected mice. The RVGL20 virus injected mice reached 50% survival point on day 65 and showed significantly longer survival time up to 116 days. The single mutant TK-virus of LIVP virus was less pathogenic than the single mutant F3-or double mutant F3-, TK- viruses; all mice were alive on day 80 after injection with TK- virus and 14.3% of the mice survived 130 days. All mice injected with the triple mutant TK-, F3-, and HA-virus (RVGL21) survived 130 days (duration of the experiment) and continued to live without any signs of virus toxicity compared to other groups of mice.

Splenomegaly in various mice

Immunocompetent C57BL/6 mice

Several groups of the animals demonstrated enlargement of the spleen; therefore the relative spleen weight (RSW) was calculated. The results are shown in Table 2 as follows:

Table 2. Relative spleen weight (RSW) in mice with or without tumors.

Groups	Glioma model nu/nu mice	Breast cancer model nu/nu mice	Melanoma model C57BL/6 mice
No tumor, PBS	43.6 ± 4.1[a]	50.5 ± 11.2[d]	30.1 ± 2.8[g]
No tumor, LIVP	67.2 ± 11.9	48.0 ± 13.1	68.1 ± 9.4
Tumor, PBS	92.4 ± 7.4[b]	84.1 ± 14.6[e]	106.0 ± 46.1[h]
LIVP	98.2 ± 28.2[c]	108.4 ± 39.4[f]	148.4 ± 44.8[i]
RVGL2	96.0 ± 34.9	112.7 ± 15.6	51.9 ± 6.6

Groups	Glioma model nu/nu mice	Breast cancer model nu/nu mice	Melanoma model C57BL/6 mice
RVGL5	143.8 ± 20.5	169.6 ± 31.7	61.6 ± 2.9
RVGL9	73.9 ± 10.5	151.8 ± 27.9	63.3 ± 34.9
RVGL20	84.9 ± 6.6	159.9 ± 22.7	106.7 ± 36.0
RVGL21	114.4 ± 12.5	117.7 ± 15.3	63.0 ± 24.6
WR	37.3 ± 3.5	57.9 ± 10.9	70.5 ± 1.8
RVGL23	46.9 ± 15.7	73.1 ± 19.3	97.0 ± 43.9

Mean ± SD for n=4-8 mice/group.
RSW = weight of spleen (g) x 10^4/(animal body weight (g) – tumor weight (g)).
[a] $p \leq 02.02$ vs. all groups, except no tumor LIVP, WR, RVGL23
[b] $p \leq 0.039$ vs. no tumor PBS, no tumor LIVP, RVGL5, WR, RVGL23
[c] $p \leq 0.046$ vs. all groups, except PBS, RVGL2, RVGL20, RVGL21
[d] $p \leq 0.006$ vs. all groups except no tumor LIVP, PBS, WR, RVGL23
[e] $p \leq 0.048$ vs. all groups, except no tumor PBS, LIVP, RVGL2, WR, RVGL23.
[f] $p \leq 0.045$ vs. all groups, except PBS, RVGL2, RVGL21
[g] $p \leq 0.035$ vs. PBS, LIVP, RVGL20, WR, RVGL23
[h] $p \leq 0.049$ vs. all other groups, except no tumor LIVP, RVGL20, WR, RVGL23
[i] $p \leq 0.049$ vs. all other groups.

As shown in the Table 2 above, some degree of splenomegaly was observed in mice. For immunocompetent C57BL/6 mice, a statistically significant difference ($p < 0.035$) was found in tumorous mice injected with PBS, LIVP, RVGL20, WR and RVG123 compared to non-tumorous mice. In mice injected with wt VV strain LIVP spleen was enlarged greatly ($p < 0.049$) versus all other groups. In contrast, the smallest spleens were found in the mice without tumor.

Nude mice with a glioma tumor

In nude mice with or without s.c. glioma tumor, mice injected with wt WR or TK- of WR virus had the lowest RSW 37.3 or 46.9, respectively, which was similar to the RSW from the mice without tumor and injected with PBS (43.6). The largest RSW 143.8 and 114.4 was observed in RVGL5 (HA-) and RVGL21 (TK-, F3-, HA-) groups, respectively. No statistically significant difference was found among the groups of mice injected with wt LIVP, RVGL2, RVGL9, RVGL20 versus the PBS injected group.

Nude mice with breast tumor

The results of RSW in the immunocompromised mice bearing s.c human breast tumor indicate that all mice injected with wt LIVP and its mutants have an enlarged spleen compared to the mice injected with wt WR or TK- WR viruses ($p<0.045$). The largest spleen was found in the mice injected with single HA-, single F3-, double F3-, TK- mutants of LIVP strain.

Other results using RVGL21 for injection

Two mice #437 and #458 survived more then 190 days after RVGL21 injection (10^7 and $4x10^5$, respectively, i.v.) without any signs of diseases or virus related toxicities.

On day 30 after GI-101A cell implantation (tumor volume=594.9mm^3), 10^7 of RVGL21 was injected i.v. into mouse #437. On day 101 after virus injection (s.c. tumor size=220.4 mm^3), metastasis (hard tissue) in chest area under the skin was observed. The size of the tumor was 1223.6 mm^3, which disappeared by day 148. The s.c. tumor did not disappear, it started to grow back, but the mouse remained metastasis-free.

Mouse #458 had a first s.c. tumor (GI-101A) on the right hind quarter. When the first tumor started to shrink (day 29 after RVGL21 virus injection, tumor size=1924.3 mm^3), a second syngenic tumor was implanted s.c. on the left hind quarter. The second tumor grew slowly, reached the size of 1205.7 mm^3 and started to shrink. The mouse was free of the first tumor on day 127 post virus injection; the size of the second tumor was 439.6 mm^3 . The tumor continued to shrink and the cells died. The body gradually absorbed remaining tumor tissues that were contributed by the host (such as the tumor vascular skeleton that was coming from the host). Since these remains are not considered foreign, the immune system doesn't destroy them. The tumor cells, on the other hand, were long gone and cleared by the immune system and the virus. Reduction of the second syngeneic tumor demonstrates that this mouse developed antibodies against the tumor cells. The antibodies resulted in the reduction of the second syngeneic tumor.

EXAMPLE 7

Use of a Microorganism or Cell to Induce Autoimmunization of an Organism Against a Tumor

This example shows that the method provided herein and in priority application EP 03 018 478.2 relating to "The production of a polypeptide, RNA or other compound in a tumor tissue" also can be used for the production of antibodies against the tumor tissue. These antibodies provide for autoimmunization of the organism bearing the tumor. Furthermore, these antibodies can be isolated and used for the treatment of tumors in other organisms.

Methods and uses of microorganisms, including cells, which can contain DNA encoding a desired polypeptide or RNA, to induce autoimmunization of an organism against a tumor are provided. Also provided are methods for the production of antibodies against a tumor by: (a) injecting a microorganism, such as a virus or cell, optionally containing a DNA sequence encoding a desired polypeptide or RNA, into an organism bearing a tumor and (b) isolating antibodies against the tumor.

This Example further demonstrates that administration of microorganisms, such as the triple mutant vaccinia virus strain provided herein, which accumulate in tumors, causing them to release tumor antigens for a sufficient time to permit production of antibodies by the host. This is exemplified by showing a reduction and elimination of xenogeneic GI-101A solid breast carcinoma tumors and their metastases in nu-/nu- mice (T cell deficient mice).

Step#1: Female nu-/nu- mice of 5 weeks age were chosen, and the GI-101A cells grown in RPMI1640 medium, supplemented with estrogen and progesterone. The confluence was reached, cells were harvested, washed with phosphate buffered saline. Cells (5×10^6 cells per mouse) were then injected subcutaneously into mice. The tumor growth was carefully monitored every two days.

Step#2: At two stages of tumor growth (at tumor size of 400-600mm^3, and at tumor size of ~ 1700 mm^3), purified vaccinia viral particles (RVGL12) were delivered to each tumorous mice by intravenous injection through tail vein. The colony purified virus was amplified in CV-1 cell line and the intracellular viral particles were

purified by centrifugation in sucrose gradient. Two concentrations of virus (10^6 pfu/100 µl and 10^7 pfu/100 µl resuspended in PBS solution) were injected. The viral replication was monitored externally by visualization of virus-mediated green fluorescence protein expression. The tumor development was monitored by tumor volume determination with a digital caliper.

Vaccinia viruses RVGL12+GCV(gancyclovir) , and RVGL12 (RVGL12 is the same as RVGL7, except that the nucleic acid encoding gfp is replaced by herpes simplex virus thymidine kinase (HSV TK; see, SEQ ID Nos. 35 and 36) were injected 67 days after GI-101A cellular implantation. A second administration referred to as RVGL12a, was injected 30 days after cellular implantation. .

Step#3: After viral administration , it was determined that first the tumors continued to grow to a size of ~ 900 mm^3 (from 400-600 mm^3 at the time of viral injection), and to a size of ~ 2400 mm3 (from 1700 mm^3). Then the growth rate leveled off for approximately 6-8 days.

Step#4: Approximately 14 days after viral injection, the tumor volume started to decline rapidly. Forty days after viral application, all the treated animals showed more than 60% tumor regression. Sixty-five days after viral treatment and many of the animals had complete regression of tumors.

Step#5: Some of the animals were completely tumor-free for several weeks and their body weight returned to normal. RVGL-12+GCV treatment resulted in 86.3% reduction of tumor size (Day 52 after viral injection) from their peak volumes on Day 13, RVGL-12 treatment resulted in 84.5% reduction of tumor size (Day 52) from their peak volumes (Day 13). RVGL-12a treatment resulted in 98.3% reduction of tumor size (Day 89) from their peak volumes (Day 12). After PBS+GCV control treatment, the average volume of tumors were increased by 91.8% in 38 days

Step#6: The level of immune activation was determined. Sera were obtained from the animals with regressing tumors and the immune titer determined against a foreign protein (*e.g.* green fluorescent protein), vaccinia viral proteins, and GI-101A

cancer cell proteins were determined. The following antisera obtained from the following sources were used to analyze the following listed samples.

Samples:

1). Mouse cell lysate (control);

2). Purified and denatured vaccinia viral particles;

3). GI-101A tumor cell lysate;

4). Purified green fluorescent protein;

5). Purified luciferase protein;

6). Purified beta-galactosidase protein.

Antisera:

a). Antiserum from nontumorous mouse;

b). Antiserum from GI-101A tumorous mouse;

c). Antiserum from GI-101A tumorous mouse 14 days after vaccinia i.v. injection;

d). Antiserum from GI-101A tumorous mouse 65 days after vaccinia i.v. injection;

e). Antiserum from tumor-free mouse (after elimination of GI-101A tumor) 80 days after vaccinia i.v. injection.

The results showed that there was enormous tumor-specific vaccinia virus replication in the tumors, which led to tumor protein antigen and viral protein production in the tumors. In addition, the vaccinia virus did lyse the infected tumor cells thereby releasing tumor-cell-specific antigens. The continuous leakage of these antigens into the body led to a very high level of antibody titer (in approximately 7-14 days) against foreign cell proteins (tumor proteins), viral proteins, and the virus encoded engineered proteins in the mouse body. The newly synthesized antitumor antibodies and the enhanced macrophages, neutrophils counts were continuously delivered via the vasculature into the tumor and thereby providing for the recruitment of an activated immune system in the inside of the tumor. The active immune system then eliminated the tumor including the viral particles. This interconnected release of foreign antigens boosted antibody production and continuous return of the antibodies against the tumor-contained proteins function as

an autoimmunization vaccination system, initiated by vaccinia viral replication, followed by cell lyses, protein leakage and enhanced antibody production .

EXAMPLE 8

Production of β-Galactosidase and Anti β-Galactosidase via Vaccinia Virus Delivered lacZ in Tumor Bearing Mice

Thirty five athymic nu/nu mice (5 weeks old, 25g, male) were used to demonstrate the biodistribution and tumor targeting of vaccinia virus (strain LIVP) with different deletions in the genome. Mice were divided into 7 groups with 5 in each group as presented in Table 1

Group	No. mice	Tumor implanted	Virus Injected	Insertion locus
1	5	None	VGL	wtLIVP
2	5	C6, s.c. 5 x 10^5 cells	VGL	wtLIVP
3	5	C6, s.c. 5 x 105 cells	RVGL1	N-*luc*, *lacZ*
4	5	C6, s.c. 5 x 105 cells	RVGL5	HA- *lacZ*
5	5	C6, s.c. 5 x 105 cells	RVGL7	TK-*egfp*, *lacZ*
6	5	C6, s.c. 5 x 105 cells	RVGL8	*Not*I-*lacZ*
7	5	C6, s.c. 5 x 105 cells	RVGL19	TK-*rTrf*, *lacZ*, *Not*I-RG

C6 gliomas were subcutaneously developed in Groups 2 to 7. Five days after tumor cell implantation ($5x10^5$ cells/mouse), each animal was treated with 0.1 ml of virus at a multiplicity of infection (MOI) of $1x10^7$ via tail vein injection. Two weeks after virus injection, all mice were sacrificed and blood samples were collected. Various organs and tumors also were taken from animals for virus titer and β-galactosidase analysis.

The β-galactosidase analysis was performed using the Galacto-Light Plus system (Applied Biosystems), a chemiluminescent reporter gene assay system for the detection of β-galactosidase, according to the manufacturer's instructions.

β-galactosidase Expression Measurements

In non-tumorous mice as well as in tumorous mice injected with wild type vaccinia virus (without reporter genes and without β-galactosidase gene) no β-galactosidase expression was detected in organs, blood and tumor samples. By contrast, in the tumors of mice infected with β-galactosidase expressing virus, high

levels of β-galactosidase was expressed. β-galactosidase also was detected in blood samples as shown in Table 2, but no virus was recovered from blood samples.

Table 2. Production of β galactosidase by vaccinia virus in tumor and blood from tumor bearing mice (day 14 after virus injection)

Group	Virus Injected	β-gal in tumor µg/mg of total protein	β-gal in serum µg/ml of total protein	Est. total β-gal/tumor (µg)	Est. total β-gal/5ml blood (µg)
3	RVGL1	1.59 ± 0.41	$1.38x10^{-2}$±$1.09x10^{-2}$	489.84	4.00
4	RVGL5	1.51 ± 0.37	$1.16x10^{-2}$±$1.08x10^{-2}$	330.21	3.62
5	RVGL7	1.35 ± 0.59	$0.95x10^{-2}$±$1.47x10^{-2}$	616.60	1.83
6	RVGL8	1.81 ± 0.42	$0.86x10^{-2}$±$0.33x10^{-2}$	962.36	2.38
7	RVGL19	1.30 ± 0.44	$0.26x10^{-2}$±$0.16x10^{-2}$	463.75	0.60

Anti-β-galactosidase antibody production

To determine whether the amount of β-galactosidase presented in mouse blood was sufficient to elicit antibody production, sera taken from two mice (mouse #116 from Group 5, and #119 from Group 6) were collected and tested for primary antibodies against β-galactosidase in Western analysis. β-galactosidase from *E. coli* (Roche, 567 779) was used as the antigen standard, and the mouse monoclonal anti β-galactosidase from E. coli (Sigma, G6282) was used as the antibody positive control. As additional sources of β-galactosidase, total protein was obtained from CV-1 cells 24 hours after infection with RVGL7 at MOI of 1 pfu/cell, and the tumor protein sample from mouse designated #143 (treated with RVGL7) was obtained.

The protein samples were prepared in triplicate, each set including a β-galactosidase antigen control, a cell lysate from RVGL7 infected CV-1 cells, and tumor lysate from mouse #143. All protein samples were separated by electrophoresis using a 10% polyacrylamide gel, and transferred to NitroBind nitrocellulose membrane (MSI) using a BioRad semidry blotting system. Immunoblotting was performed with either 1:3000 mouse monoclonal anti β-galactosidase, or 1:3000 mouse serum taken from either mouse #116 or #119, and 1:3000 Goat AntiMouse IgG-HRP (BioRad). An Amplified Opti-4CN Detection Kit (BioRad) was used for detection.

The results showed that sera taken from mouse #116 and #I19 exhibited similar levels of antibody when compared to a commercial mouse anti-β-

galactosidase standard , and demonstrated that the tumor bearing mice #116 and #119 produced antibodies against β-galactosidase.

EXAMPLE 9

Mammalian cells for tumor therapy

As shown herein, certain bacteria, viruses, and mammalian cells (BVMC), when administered systemically, again enter and selectively replicate in tumors. Hence, systemically injected mammalian cells and certain bacterial (anaerobic bacteria, such as Salmonella, Clostridium sp., Vibrio, *E. coli*) cells gain entry into solid tumors and replicate in tumor-bearing organisms. Genetically-labeled cells can be used for tumor detection and therapy. In addition to gene expression in tumors through BVMC targeting, tumor-specific gene expression can be achieved by linking transgenes to tissue/tumor-specific promoters. To obtain tumor specific gene expression, a variety of systemic targeting schemes can be employed. These strategies include the use of tissue/tumor-specific promoters that allow the activation of gene expression only in specific organs, such as prostate-specific promoter-directed viral gene expression; the use of extracellular matrix (*i.e.* collagen)-targeted viral vectors; and the use of antibody-directed viral vectors. Conditionally-replicating viruses have also been explored as tumor-specific delivery vehicles for marker genes or therapeutic genes, such as oncolytic adenovirus vector particles, replication-selective HSV, vaccinia viruses and other such viruses.

When light-emitting protein encoded BVMC are injected systemically into rodents, tumor-specific marker gene expression is achieved and is detected in real time based on light emission. Consequently, the locations of primary tumors and previously unknown metastases in animals are revealed in vivo. Hence diagnosis can be coupled to therapy and to monitoring of therapy. The impaired lymphatic system in tumors may be responsible for the lack of clearance of bacteria from tumors by the host immunosurveillance after escaping the vascular system.

EXAMPLE 10

Tumor Development is inhibited following S.pyogenes administration

This Example and following examples demonstrate the use of bacterial cells to colonize tumors, use of reporter in the cells to quantitate colonization; use of the colonized attenuated bacterial cells for tumor inhibition. Co-administration or sequential administration of bacteria and viruses. Admistration of virus before bacteria increase tumor colonization by the bacteria. Administer bacteria that expresses an enzyme that will activate a prodrug, thereby targeting colonized cells.

Bacterial Strains

Streotococcus pyogenes M-type 1 T-type 1 (ATCC catalog no. 700294) was transformed with pDC123-luxF plasmid) that contains the bacterial luciferase expression cassette (Lamberton GR, Pereau MJ, Illes K, Kelly IL, Chrisler J, Childers BJ, Oberg KC, Szalay AA. 2002. *Construction and characterization of a bioluminescent Streptococcus pyogenes.* Proceedings of the 12th International Symposium on Bioluminescence and Chemiluminescence, Case JF, Herring PJ, Robison BH, Haddock SHD, Kricka LJ, Stanley PE (eds). Chichester: Wiley, pp 85-88. Luciferase can be detected in the presence of exogenous decanal.

Transformed S.pyogenes were grown overnight in BH1 media in the presence of 20 μg/ml of chloramphenicol at 37°C. After overnight growth, the bacteria were counted at OD_{600} and bacteria were resuspended in BH1 media at the indicated density for injection.

Tumor Development and Bacterial Injection

Twenty 5-week old mice were injected subcutaneously in the right lateral thigh. Each mouse was injected with 5×10^5 C6 glioma cells transformed with pLEIN-derived retrovirus (Clontech; see also WO 03/14380). The subcutaneous tumors were developed for 7 days after implantation before bacterial injection.

For bacterial injection, the tumor-bearing mice were anesthetized with isofluorane. The suspensions were injected intravenously with a 1-cc insulin syringe equipped with a 29 ½ -gauge needle through a surgically exposed femoral vein. After the injections, the incisions were sutured.

Tumor growth was monitored twice a week following bacterial injection using a digital caliper. In addition, fluorescence imaging and photographic images

of the animals were taken at the end time points. The presence of luminescent bacteria was analyzed by intravenously injecting the animals with 30 µl of decanal. Analysis of whole animals for bacterial luciferase activity, followed methods similar to Yu *et al.* (2004) *Nature Biotechnology 22(3)*: 313-20. Briefly, anesthetized animals were placed inside the dark box for photon counting (ARGUS 100 low light Imager, Hamamatsu). Photon collection was for 1 minute from ventral and dorsal sides of the animal and the images were recorded with Image Pro Plus 3.1 software (Media Cybernetics) and/or Lighttools® macroimaging system. A light image also was recorded. The luminescent images were superimposed on the light image to localize the luminescent activity on the animal. Total intensity of photon emission in localized regions, *e.g.* in the tumor region, also was recorded. S.pyogenes was isolated from removed tumors and ground tissue was plated on LB-chloramphenicol (20 µg/ml) plates. Luminescent bacteria were counted in the presence of decanal vapor.

Results

Four groups of mice were tested. Each group contained five mice.

Group	*S.Pyogenes*
1	None
2	1×10^6
3	1×10^7
4	5×10^7

Tumor volume was measured after 7 days of tumor development and the injection of S.pyogenes, through 21 days post-tumor development.

The control group of mice with no S.pyogenes had continuous and accelerating tumor growth over the 2-week period. The mice injected with *S.* pyogenes had slower tumor growth. Groups 3 and 4 had the slowest tumor growth rates. Both groups maintained a slower linear rate throughout the monitoring period, whereas the control group, not injected with bacteria, exhibited tumor growth that accelerated at later time periods.

At all time points following bacterial injection, tumor volumes were smaller in Groups 3 and 4 mice than in the control mice (Group 1). At day 21, the average

tumor volume of the control group was approximately 2.5-3 fold greater than the average tumor volumes in Groups 3 and 4. Group 2, injected with the lowest titer of bacteria, also had a reduced tumor volume from the control group at the later time points, although the tumor volume was larger than Groups 3 and 4.

Bacterial colonization and tumor inhibition also is assayed in a fibrosarcoma model. HT1080 fibrosarcoma cells transformed with the pLEIN retrovirus are injected subcutaneously into the right lateral thigh of five week old nude male mice 5×10^5 cells/mouse). S. pyogenes transformed with pDC123-luxF is injected into the femoral vein of the animals after 8 or 14 days of tumor growth (5 animals on each day). A group of 5 animals are not injected as serve as a control group. Tumor growth and luciferase activity is monitored at subsequent time points. S.pyogenes is isolated from tumors and cultured on BH1 + chloramphenicol (20 μg/ml) plates. Luminescent bacterial colonies are counted in the presence of decanal vapor.

Example 11

Vibrio Cholera localization to tumors

Plasmids and Bacterial Strains

Attenuated *Vibrio Cholerae*, strain Bengal 2 serotype 0139, M010 DattRS1, was transformed with pLITE201 which contains the luxCDABE cassette (Voisey *et al.* (1998) *Biotechniques 24*:56-58). The transformed strain is a light emitting strain due to the expression of the luciferase genes.

Tumor Development and Bacterial Injection

Groups of nude mice (n>20) were implanted with C6 glioma tumors ($500mm^3$) as described in the Examples herein. 1×10^8 transformed bacteria (*V.Cholerae)* were suspended in 100 μl of phosphate buffered saline (PBS). The bacterial suspension was injected into the right hind leg of each mouse. The animals were then monitored after injection under a low light imager as described in Example A.

In a separate experiment, for comparison, groups of nude mice (n>20) were implanted with C6 glioma tumors ($500mm^3$) as described in the Examples herein.

These mice were injected with 1x 10^8 pfu/mouse of rVV-RUC-GFP virus (see Examples 1 and 4).

Results

Titer and luciferase activity

Mice from each of the two injected groups were sacrificed at time points after injection. Tumors were excised and homogenized. Bacterial and viral titers and luciferase activities were measured as described in the Examples herein.

Both bacterial and viral titer increased following injection. The increase in bacterial growth over time was proportional to luciferase levels in the tumors. A log-log plot of bacterial titer versus luciferase activity in tumors in the mice injected with *V. cholera* demonstrated a linear relationship between bacterial titer and luciferase activity. The groups of mice injected with rVV-RUC-GFP virus, also demonstrated a linear relationship between virus titer and luciferase activity.

	Time after *V. Cholera*/pLITE injection			
	4 hrs	**8 hrs**	**16 hrs**	**32 hrs**
Bacterial Titer (cfu/tumor)	3.79 X 10^4 $\pm$2.93	3.14 X 10^6 $\pm$2.45	1.08 X 10^8 $\pm$1.3	5.97 X 10^8 $\pm$4.26

	Time after rVV-ruc-gfp virus injection			
	36 hrs	**Day 3**	**Day 5**	**Day 7**
ViralTiter (pfu/tumor)	3.26 X 10^6 $\pm$3.86	7.22 X 10^7 $\pm$3.67	1.17 X 10^8 $\pm$0.76	3.77 X 10^8 $\pm$1.95

The experiments demonstrated a linear relationship between titer and luciferase activity. Thus, luciferase activity of the injected bacteria and/or virus can be used a correlative measurement of titer.

Localization

Localization of *V.cholera* was performed as detailed in the Examples herein for virus. Briefly, organs and blood samples were isolated from animals euthanized

with CO_2 gas. The organs were ground and plated on agar plates with chloramphenicol drug selection for analysis of bacterial titer.

Bacterial titer was assayed in tumor, liver, testes, spleen, kidney, lung, heart, bladder and brain of the injected mice. Samples were taken from mice sacrificed at zero, and subsequent times up to 150 hours following *V.cholera* injection.

At the time point immediately following injection (t=0), *V. cholera* was present in all samples, with the highest levels in the liver and spleen. By 50 hours post-injection, titer of *V.cholera* in all tissues had reduced with the exception of tumor tissue. In contrast, *V.cholera* titer had increased about 4 orders of magnitude as compared to time zero. This level increased slightly and then stayed constant throughout the remainder of the experiment. By 150 hours post-infection, titer in all samples except tumor had decreased. For example, the titer in liver had decreased by approximately 5 orders of magnitude from the time zero point. At the 150 hour point, the *V.cholera* titer in the tumor tissue was about 6 orders of magnitude greater than any other tissue sample.

Example 12

Co-administration and sequential administration of bacteria and virus

V.Cholera/pLITE (see Example B) and vaccinia virus VV-TK$^-$-gfp-lacZ (see Example 4) were administered together or sequentially. Groups of nude mice with C6 glioma tumors were injected with bacteria and/or virus as shown in the Table below. Three male mice were injected per group. Bacteria and/or virus were injected on day 11 and day 16 following tumor implantation. Tumor growth, luciferase and GFP activity were monitored as described in the Examples herein.

Group	Day 11 injection	Day 16 injection
1	1 X 10^7 VV-TK$^-$-gfp-lacZ	1 X 10^7 *V.Cholera*/pLITE
2	None	1 X 10^7 *V.Cholera*/pLITE
3	1 X 10^7 *V.Cholera*/pLITE	1 X 10^7 VV-TK$^-$-gfp-lacZ
4	None	1 X 10^7 VV-TK$^-$-gfp-lacZ
5	None	1 X 10^7 VV-TK$^-$-gfp-lacZ and 1 X 10^7 *V.Cholera*/pLITE

Results

On day 21 (21 days post tumor implantation) animals were sacrificed. Tumors were excised from each animal and ground. Viral titer was assayed on Groups 3, 4 and 5. Bacterial titer was assed on Groups 1,2 and 5. Titers (colony forming units and plaque forming units) were performed as previously described in the Examples.

A comparison of the bacterial titer in tumors Groups 1, 2 and 5 demonstrated that bacterial titer was highest in Group 1 that had been injected first with vaccinia virus at day 11, and followed by *V.cholera* injection on day 16. Co-injection of bacteria and virus at day 16 (Group 5) gave an intermediate bacterial titer. Group 2, injected only with *V.cholera* at day 16, had a lower bacterial titer in the tumor tissue than either of groups 1 or 5. Thus, tumors were more susceptible to bacterial colonization when first colonized by VV-TK$^-$-gfp-lacZ virus.

A comparison of the viral titer in Groups 3, 4 and 5 demonstrated that Group 4, with only virus injection at day 16, had the highest viral titer followed by Groups 5 and 3. The viral titer of Group 5 was slightly higher than Group 3, but not apparently significantly different. One mouse in Group 4 had a viral titer that was an extreme outlier in comparison to the viral titer of the other 2 mice in Group 4. When the numbers were reassessed without this mouse, the general trend remained the same. The average viral titer in Group 4 was much closer to the viral titers of Groups 3 and 5. The data from the three groups in this analysis was not significantly different. Thus, pre-administration of bacteria followed by administration of virus did not significantly change the viral colonization of the tumor as compared with viral administration alone.

Example 13

Tumor Inhibition by Administering PNP-expressing bacteria and prodrug

Plasmids pSOD-DeoD contains the bacterial purine nucleoside phosphorylase gene (PNP) (Sorcher *et al.* (1994) GeneTher. 1(4):223-238), under the control of the constitutive SOD (superoxide dismutase) promoter. Plasmid pSOD-DeoD-lux,

contains the luxCDABE expression cassette (Voisey *et al.* (1998) *Biotechniques 24*:56-58) inserted into pSOD-DeoD.

PNP converts the non-toxic prodrug 6-methylpurine deoxyribose (6-MPDR) to 6-methyl purine which inhibits DNA replication, transcription and translation (Sorcher *et al.* (1994) *GeneTher. 1(4)*:223-238).

Tumor Growth inhibition

Nude mice were injected with pLEIN retrovirus transformed C6 glioma cells. The pLEIN retrovirus expresses EGFP under the control of the viral promoter LTR (Clontech; see also WO 03/14380). *E. coli* DH5α expressing the bacterial purine nucleoside phosphorylase gene was injected at day 8 following tumor implantation with or without prodrug (6-methylpurine deoxyribose (6-MPDR)). Tumor volume was monitored at subsequent time points (as performed in previous examples).

Group	Administered
1	*E.coli*/PNP + prodrug
2	*E.coli*/PNP
3	*E.coli* control + prodrug

Groups 2 and 3 exhibited equal tumor growth over time points from 8 to 21 days post tumor implantation. Group 1, which received both the *E.coli* expressing PNP and the prodrug exhibited ~20% reduction in tumor size as compared to the control Groups 2 and 3 at the end time points.

To further test bacterial colonization and prodrug effects on tumor growth, a human breast cancer model, GI-101A adenocarainoma in nude mice, was chosen. GI-101A was derived from GI-101. GI-101 originated from a local first recurrence of an infiltrating duct adenocarainoma (stage IIIa, T3N2MX) in a 57 year old female patient by researchers at Rumbaugh-Goodwin Institute for Cancer Research. In the subcutaneous xenograft nude mice model, the tumor consistently metastasizes to the lungs. The GI-101A is a slower growing tumor model as compared to the C6 glioma tumor model.

Fifteen 4 week old female nude mice are each injected subcutaneously in the right lateral thigh with GI-101A cells. Thirty days after tumor development, bacteria are injected. *Escherichia coli* DH5α is transformed with pSOD-DeoD or pSOD-DeoD-lux. The bacteria are grown overnight in LB media in the presence of 20 μg/ml of chloramphenicol at 37°C. After overnight growth, the bacteria are counted at OD_{600} and bacteria resuspended in BH1 media at the indicated density. The suspensions are injected intravenously with a 1-cc insulin syringe equipped with a 29 ½ -gauge needle into the animal through a surgically exposed vein or as otherwise indicated. After the injections, the incisions are sutured.

Prodrug is administered to groups of mice every four days following injection of bacteria. Tumor growth is monitored twice per week using a digital caliper. Luciferase imaging is performed as described in the Examples herein. At the end point, the animal are sacrificed and organs are assayed as described in Example B. Histological analyses are performed to determine the degree of tumor necrosis due to bacterial colonization and/or drug treatment.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

CLAIMS:

1. A method for production of a polypeptide or RNA or a product compound, comprising:

administering to a tumor bearing non-human animal a microorganism that is a virus or a bacterium that contains nucleic acid encoding the polypeptide, encoding RNA or products in a biochemical pathway for producing the product compound, wherein:

the tumor is a solid tumor; and

the virus or bacterium accumulates in tumor cells in the animal;

harvesting tumor tissue from the animal; and

isolating the polypeptide or RNA or compound from the harvested tumor tissue

2. A method for simultaneously producing a polypeptide, RNA molecule or cellular compound and an antibody that specifically reacts with the polypeptide, RNA molecule or compound, comprising:

(a) administering a virus or a bacterium that contains nucleic acid encoding the polypeptide, encoding RNA or a pathway for producing the product compound to a tumor bearing non-human animal, wherein:

the tumor is a solid tumor; and

the virus or bacterium accumulates in tumor cells in the animal;

(b) isolating an antibody that specifically reacts with the polypeptide, RNA molecule or compound from serum that was obtained from a tumor-bearing animal;

(c) harvesting tumor tissue from the animal; and

(d) isolating the polypeptide, RNA molecule or cellular compound from the harvested tumor tissue.

3. A method for autoimmunization of a non-human animal against a tumor to produce autoantibodies against the tumor, comprising:

(a) providing a non-human animal bearing a tumor that expresses the antigen against which antibodies are desired;

(b) administering a virus or a bacterium to the tumor bearing non-human animal, wherein:

the virus or bacterium accumulates and replicates in tumor cells in the animal to lyse the tumor cells and release tumor antigens from the tumor, whereby the animal produces autoantibodies against antigens from the tumor; and

(c) isolating autoantibodies from serum.

4. The method of any of claims 1-3, wherein the animal is a rodent.

5. The method of claim 4, wherein the rodent is a mouse.

6. The method of claim 4, wherein the rodent is a rat.

7. The method of any of claims 1-6, wherein the microorganism-is an attenuated bacterium.

8. The method of claim 7, wherein the bacterium is selected from among attenuated Vibrio cholerae, E. coli, listeria, salmonella and streptococcus strains.

9. The method of claim 8, wherein the virus is selected from among pox viruses, herpes viruses, adenoviruses and sindbis virus.

10. The method of claim 9, wherein the virus is an attenuated cytoplasmic virus.

11. The method of claim 9, wherein the virus is an attenuated pox virus.

12. The method of claim 9, wherein the virus is a vaccinia virus.

13. The method of claim 12, wherein the vaccinia virus is a Lister strain.

14. The method of claim 12, wherein the vaccinia virus is a LIVP strain.

15. The method of any of claims 1-14, wherein the microorganism comprises a reporter gene construct.

16. The method of claim 15, wherein the reporter gene construct encodes a detectable protein or a protein that induces or produces a detectable signal.

17. The method of claim 16, wherein the protein is a luciferase or a fluorescent protein.

18. The method of claim 15, wherein the reporter gene construct encodes a bioluminescence generating system and optionally encodes a fluorescent protein.

19. The method of claim 16 or claim 17, wherein the detectable protein or protein that induces or produces a detectable signal is to be detected for monitoring the localization and/or level of the microorganism in the non-human animal.

20. The method of claim 19, wherein detection is effected with a low light imaging device.

ABSTRACT

5

MODIFIED RECOMBINANT VACCINIA VIRUSES AND OTHER MICROORGANISMS, USES THEREOF

10 Recombinant *vaccinia* viruses useful as tumor-specific delivery vehicle for cancer gene therapy and vaccination Therapeutic methods and microorganisms therefore are provided. The microorganisms are designed to accumulate in immunoprivileged tissues and cells, such as in tumors and other proliferating tissue and in inflamed tissues, compared to other tissues, cells and organs, so that they exhibit relatively low toxicity to host organism. The microorganisms also are 15 designed or modified to result in leaky cell membranes of cells in which they accumulate, resulting in production of antibodies reactive against proteins and other cellular products and also permitting exploitation of proliferating tissues, particularly tumors, to produce selected proteins and other products. Methods for making tumor specific antibodies and also methods of making gene products encoded by the microorganism as well as antibodies reactive therewith are 20 provided.

(No suitable figure)

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<212> DNA
<213> Vaccinia Virus/Ankara

<300>
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<309> 2003-04-14

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Tyr

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<212> DNA
<213> Vaccinia Virus/Tian Tan

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<212> PRT
<213> Vaccinia Virus/Tian Tan

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<212> PRT
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Met Ala Ser Tyr Pro Cys His Gln His Ala Ser Ala Phe Asp Gln Ala
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Ala Arg Ser Arg Gly His Asn Asn Arg Arg Thr Ala Leu Arg Pro Arg
            20   25   30
Arg Gln Gln Lys Ala Thr Glu Val Arg Leu Glu Gln Lys Met Pro Thr
        35   40   45
Leu Leu Arg Val Tyr Ile Asp Gly Pro His Gly Met Gly Lys Thr Thr
    50   55   60
Thr Thr Gln Leu Leu Val Ala Leu Gly Ser Arg Asp Asp Ile Val Tyr
65   70   75   80
Val Pro Glu Pro Met Thr Tyr Trp Arg Val Leu Gly Ala Ser Glu Thr
                85   90   95
Ile Ala Asn Ile Tyr Thr Thr Gln His Arg Leu Asp Gln Gly Glu Ile
            100   105   110
Ser Ala Gly Asp Ala Ala Val Val Met Thr Ser Ala Gln Ile Thr Met
        115   120   125

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Gly Met Pro Tyr Ala Val Thr Asp Ala Val Leu Ala Pro His Ile Gly
    130                 135                 140
Gly Glu Ala Gly Ser Ser His Ala Pro Pro Pro Ala Leu Thr Leu Ile
145                 150                 155                 160
Phe Asp Arg His Pro Ile Ala Ala Leu Leu Cys Tyr Pro Ala Ala Arg
                165                 170                 175
Tyr Leu Met Gly Ser Met Thr Pro Gln Ala Val Leu Ala Phe Val Ala
            180                 185                 190
Leu Ile Pro Pro Thr Leu Pro Gly Thr Asn Ile Val Leu Gly Ala Leu
        195                 200                 205
Pro Glu Asp Arg His Ile Asp Arg Leu Ala Lys Arg Gln Arg Pro Gly
    210                 215                 220
Glu Arg Leu Asp Leu Ala Met Leu Ala Ala Ile Arg Arg Val Tyr Gly
225                 230                 235                 240
Leu Leu Ala Asn Thr Val Arg Tyr Leu Gln Gly Gly Gly Ser Trp Arg
                245                 250                 255
Glu Asp Trp Gly Gln Leu Ser Gly Ala Ala Val Pro Pro Gln Gly Ala
            260                 265                 270
Glu Pro Gln Ser Asn Ala Gly Pro Arg Pro His Ile Gly Asp Thr Leu
        275                 280                 285
Phe Thr Leu Phe Arg Ala Pro Glu Leu Leu Ala Pro Asn Gly Asp Leu
    290                 295                 300
Tyr Asn Val Phe Ala Trp Ala Leu Asp Val Leu Ala Lys Arg Leu Arg
305                 310                 315                 320
Pro Met His Val Phe Ile Leu Asp Tyr Asp Gln Ser Pro Ala Gly Cys
                325                 330                 335
Arg Asp Ala Leu Leu Gln Leu Thr Ser Gly Met Val Gln Thr His Val
            340                 345                 350
Thr Thr Pro Gly Ser Ile Pro Thr Ile Cys Asp Leu Ala Arg Thr Phe
        355                 360                 365
Ala Arg Glu Met Gly Glu Ala Asn
    370                 375
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