Title: ORAL DELIVERY OF A VACCINE TO THE LARGE INTESTINE TO INDUCE MUCOSAL IMMUNITY

Abstract: Immunogenic compositions formulated for oral delivery are disclosed. These compositions include a polymer core encapsulating a target immunogen and one or more immunostimulatory agents; and a pH-sensitive copolymer matrix disposed about the polymer core. In some examples the target immunogen is an antigen derived from a viral, bacterial, fungal or parasitic pathogen. In some examples, the one or more immunostimulatory agents is a Toll like receptor ligand. Methods of inducing a mucosal immune response in the large intestine of a subject are also disclosed, such methods include administering a therapeutically effective amount of the immunogenic compositions formulated for oral delivery. Also disclosed are methods of treating or inhibiting an infection by a pathogenic bacteria, virus, fungus or parasite.

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ORAL DELIVERY OF A VACCINE TO THE LARGE INTESTINE TO INDUCE MUCOSAL IMMUNITY

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S Provisional Application No. 61/238,361, filed August 31, 2009, which is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

This disclosure relates to oral drug delivery, specifically to oral delivery of controlled release nanoparticles encapsulating a vaccine to the large intestine to induce mucosal immunity, including a protective immune response.

BACKGROUND

Sexually transmitted diseases (STDs) such as Human Immunodeficiency Virus (HIV), Human Papillomavirus (HPV), Herpes Simplex Virus (HSV), among many others, have put a significant burden on the health care of many countries, especially in the developing world. Vaccination of high-risk individuals to develop their own immune protection is the most cost-effective way to control infections and prevent disease from spreading. Effective vaccination relies largely on a robust vaccine, including powerful immune adjuvants, and a proper administration route.

Like most infectious diseases, STD infectious agents are naturally transmitted through mucosal surfaces. Vaccination by a mucosal route to induce mucosal protection has been shown more effective than by a systemic route. For example, vaccination in the lower gastrointestinal tract (such as by intrarectal administration) is an effective route for vaccination against STDs. A single dose of intrarectal immunization with a replication-deficient adenovirus expressing herpes virus glycoprotein antigen can protect from lethal challenge of HSV-2 via either colonic or genital mucosa. This route of immunization is also more effective than other mucosal routes studied.
However, many challenges remain with delivery of vaccines to the large intestine for induction of both gastrointestinal and genitourinary mucosal immunity. Vaccination of humans via the intrarectal or intracolorectal route is considered impractical and invasive. Additionally, delivery through parenteral routes would not be expected to deliver any given vaccines, such as non-live organisms, antigens and adjuvants in form of protein or peptide, to the large intestine immune system. Moreover, a simple oral vaccination has been shown to be ineffective at providing protection at the colorectal or genital mucosal because the antigens are enzymatically degraded and absorbed during the passage through the stomach and small intestine prior to reaching the lower gastrointestinal tract.

**SUMMARY OF THE DISCLOSURE**

Disclosed herein is an oral vaccination system that allows site-specific delivery of a vaccine to the large intestine as well as an enhanced immune response induced by the vaccine once delivered to the target site.

Disclosed herein is an immunogenic composition that promotes efficient particle uptake at the large intestinal mucosal surface and provides immune protection of rectal and vaginal mucosa. The composition utilizes a polymer to encapsulate a target immunogen, such as an antigen from a pathogen of interest and a pH-sensitive coating (such as EUDRAGIT®, EUDRAGIT is a registered trademark of Evonik Rohm GmbH, Pharma Polymers, Darmstadt, Germany) to protect the encapsulated particles from degradation and uptake prior to reaching the large intestine. An immunostimulatory agent, such as a Toll-like receptor ligand, is also included within the immunogenic composition and encapsulated with the target antigen by the polymer to further enhance the immune response induced by the target immunogen.

Methods are disclosed for treating and/or inhibiting an infection by a pathogen of interest in a subject. The methods include selecting a subject for treatment that has, or is at risk for developing, an infection by a pathogen of interest. The subject is administered a therapeutically effective amount of a disclosed immunogenic composition into the upper gastrointestinal tract (for example by oral, gastric or duodenal administration), wherein the target immunogen in the
immunogenic composition comprises one or more antigens present in the pathogen of interest. Oral administration is particularly advantageous because of its non-invasive nature. In some examples, the methods are methods of treating or inhibiting a sexually transmitted disease or a mucosal cancer.

Also disclosed are methods of inducing a mucosal immune response in the large intestine of a subject by oral administration of the disclosed immunogenic compositions.

In some embodiments, an immunogenic composition formulated for oral administration includes nanoparticles and a microparticle carrier encapsulating the multiple nanoparticles. In some examples, the nanoparticles include a polymer core encapsulating a therapeutically effective amount of a target immunogen and a therapeutically effective amount of one or more immunostimulatory agents. In certain examples, the microparticle carrier encapsulating the multiple nanoparticles includes a pH-sensitive copolymer matrix that is insoluble in acidic media, but dissolves by salt formation above pH 6.5, thereby forming an immunogenic composition capable of eliciting an immune response in a subject in the intestine.

The foregoing and other features of the disclosure will become more apparent from the following detailed description of a several embodiments which proceeds with reference to the accompanying figures.

**BRIEF DESCRIPTION OF THE FIGURES**

**FIG. 1A** is a schematic showing an exemplary oral vaccine delivery system.

**FIG. 1B** is a schematic showing an exemplary method of oral vaccine delivery to the large intestinal mucosa. Intraluminal pH and GI ingestion travel time are indicated (distance not to scale). Mouse total GI transit time is shorter, approximately 8 hours. Nanoparticles are coated with acid-resistant EUDRAGIT® to form microparticles, which help the nanoparticles bypass the stomach low pH. The microparticle coating begins to dissolve at the highest pH point (terminal small intestine) before entering the large intestine and is completely dissolved to release contents in the large intestine. Mucosal uptake of released nanoparticles allows site-specific, local vaccination in the large intestine.
FIGS 2A-2D illustrate intrarectal (i.c.r.) delivered nanoparticles enter the large intestinal mucosa and orally delivered FS30D/PLGA nanoparticle-releasing microparticles selectively target the large intestinal mucosa for uptake. FIG. 2A is a dot plot of a flow cytometry analysis showing colorectal mucosal uptake of PLGA nanoparticles after i.c.r. delivery of PLGA/FITC-BSA nanoparticles. Cells were isolated from the colorectum 2 days after administration and measured for FITC-positive cells ($p < 0.02$ between PLGA/FITC-BSA and PBS treated; results representative of three independent studies). Left, delivery of PLGA-FITC-BSA in PBS. Right, delivery of PBS. FIG. 2B is a density plot of a flow cytometry analysis showing induction of antigen-specific colorectal mucosal T cells after i.c.r. delivery of PLGA nanoparticles encapsulating PCLUS3-18IIB and MALP2+poly(I:C)+CpG vaccine (PLGA/PeptAg+TLRL) or PLGA nanoparticles without the vaccine. Three weeks after a single immunization, colorectal cells were isolated and measured for P18-I10 specific CD8+ T cells by tetramer staining ($p < 0.01$ between PLGA/PeptAg+TLRL and PLGA alone; results representative of two independent studies). FIG. 2C is a bar graph showing gut mucosal uptake of PLGA particles after oral delivery of FS30D/PLGA or L100-55/PLGA. Cells were isolated from the small and large intestine at day 2 for measurement of FITC-positive cells ($** p < 0.02$ on white bar indicates the difference from small intestine and $*** p < 0.001$ on black bar indicates the difference from small intestine; results are representative of two independent studies). FIG. 2D is a series of representative dot plots of flow cytometry from studies tabulated in FIG. 2C.

FIG. 3A is a graph illustrating of particle size distribution of PLGA/FITC-BSA nanoparticles and a digital image of a scanning electron microscopy (SEM) image of micrographs of nanometer-size poly-lactic-co-glycolic acid (PLGA)/FITC-BSA nanoparticles. Image taken at 50,000x magnification.

FIG. 3B is a graph showing the results of tests of the dissolution of unencapsulated BSA compared to BSA-encapsulated PLGA nanoparticles. The dissolution of 100% powder (100 µg/ml) was tested using a USP dissolution bath in 10 mM PBS (pH 7.4) at a paddle speed of 6 rpm.

FIGS. 4A-4D show orally delivered FS30D coated PLGA nanoparticle vaccine induces antigen-specific T cells in the large intestine, while L100-
55/PLGA/vaccine induces the T cells in the small intestine. **FIG. 4A** is a set of dot plots of flow cytometric data showing the activation of dendritic cells (DC) after incubation with supernatants from FS30D coated PLGA containing PCLUS3-18IIIB+TLRL (FS30D/PLGA/PeptAg+TLRL), antigen peptide without TLRL (FS30D/PLGA/PeptAg), or only vaccine (PeptAg+TLRL) dissolved in PBS at pH 7.4 for 16 hours. After centrifugation at 2,000 rpm, the supernatants were incubated with bone marrow derived DCs for 20 hours. Intracellular IL-12 was measured by flow cytometry. *p < 0.01* between FS30D/PLGA/PeptAg+TLRL and FS30D/PLGA/PeptAg or Unstimulated. No difference was observed between FS30D/PLGA/PeptAg+TLRL and PeptAg+TLRL. **FIGS. 4B-4D** are density plots of flow cytometric data and a bar graph showing induction of T cell responses after oral delivery of FS30D/ or L100-55/PLGA/PeptAg+TLRL. Oral administration to BALB/c mice was conducted twice with a 2-week interval. Tetramer positive cells in the colorectum (FIGS. 4B and 4C) or upper part of the small intestine (**FIG. 4D**) were measured three weeks after the second immunization. The i.c.r. group was immunized instead with vaccine only without particles (PeptAg+TLRL, i.c.r.). ***p < 0.01, ***p < 0.001** indicate the significant difference between the group with asterisks and each of the groups without asterisks. There are no differences between the two groups with asterisks. **FIG. 4C** is representative of two independent studies and **FIG. 4B** is a representative flow pattern from **FIG. 4C**. In **FIG. 4D**, *p* value less <0.001 for L100-55 was observed as compared to the other groups. **FIGS. 5A and 5B** are bar graphs showing orally delivered FS30D coated PLGA nanoparticle peptide vaccine confers T-cell mediated resistance to virus infection in the rectal (**FIG. 5A**) or vaginal (**FIG. 5B**) tract. FS30D/ or L100-55/PLGA/PeptAg+TLRL was given orally to mice twice with a two-week interval, followed by i.c.r. (**FIG. 5A**) or i.vag. (**FIG. 5B**) challenge with 2x10^7 or 1x10^7 PFU of vPE16, respectively, three weeks after the last immunization. Ovaries (where this virus primarily replicates) were removed at day 6 for viral titer assessment. ***p < 0.01, ***p < 0.001** indicate the significant difference in viral titer between the group with asterisks and each of the groups without asterisks. **FIGS. 6A and 6B** illustrate orally delivered FS30D coated PLGA nanoparticle protein vaccine confers antibody-mediated resistance to virus infection.
in the rectal or vaginal tract. FS30D coated PLGA containing antigen proteins A33 and L1 and TLR ligands (ProtAg+TLRL) were administered orally with a two-week interval. FIG 6A is a set of bar graphs illustrating serum and local IgA (top) and IgG (bottom) antibodies against both A33 and L1 (together) measured at two weeks after the last immunization. Both FS30D/PLGA/ProtAg+TLRL p.o. and ProtAg+TLRL i.c.r. groups have significantly higher antibody titers than the other groups (p < 0.02). **p < 0.01 and ***p < 0.001 indicate difference (for both sites) from each of the bars without asterisks. FIG. 6B is a set of graphs illustrating the disease course of the mice after challenge with WR by the i.c.r. (4x10⁷ PFU) or i.vag. (1x10⁷ PFU) route three weeks after the last immunization. **p < 0.02, ***p < 0.001 indicate the differences between the FS30D/PLGA/ProtAg+TLRL and unimmunized groups in weight loss. §, 75% mortality; %, 50% mortality.

FIG. 7A is a set of dot plots of flow cytometric data showing colorectal mucosal uptake of PLGA nanoparticles administered i.c.r. Two days after administration of PLGA/FITC-BSA nanoparticles, cells were isolated from the lamina propria and gated for all cells (each left panel) or FITC⁺ cells (each right panel) from untreated mice (left group) or mice receiving particles (right group) after staining for CD11b, CD11c, and B220. Numbers for each region are the percentage of all cells (including FITC⁺ and FITC⁻).

FIG. 7B is a transmission electron microscopy (TEM) image of intracellular PLGA nanoparticles after i.c.r. delivery of PLGA nanoparticles (left, 2,800x; right, 6,400x). ER: endoplasmic reticulum; N, nucleus; n, nucleolus; NM, nucleus membrane; PM, plasma membrane.

FIGS. 8A-8C illustrate uptake of PLGA nanoparticles by mouse bone marrow derived dendritic cells and colorectal mucosal cells. FIG. 8A is a set of histograms of flow cytometry measuring fluorescence expression. Bone marrow derived dendritic cells (DCs) were incubated with PLGA/FITC-BSA at different doses for 24 hours before the assay. FIG. 8B is a set of digital images of light microscopy (20x) to locate cells (left) and fluorescent microscopy to visualize FTIC-containing particles (right, photo enhanced). FIG. 8C is a TEM image of PLGA particles (marked with arrows) in the cytoplasm of dendritic cells after incubation with the particles (2,800x).
FIGS. 9A and 9B illustrate EUDRAGIT® FS30D size distribution and dissolution. FIG 9A is a tracing illustrating particle size before (top) and after 1 hour (bottom) incubation with PBS at pH 7.4. FIG. 9B is a TEM image of FS30D released PLGA. FS30D/PLGA were incubated for 1.5 hours in PBS at pH 7.4 (4,600x), and supernatants were concentrated for TEM (left). The particle diameter was measured using ImageJ. The geometric mean and size range are shown in the bar graph (right).

FIGS. 10A and 10B illustrate FS30D microparticles release PLGA nanoparticles at a high pH. FIG. 10A is a graph illustrating PLGA/FITC-BSA nanoparticle release from FS30D microparticles determined by UV absorption at 494 nm. FIG. 10B is a fluorescence image of PLGA/FITC-BSA released from FS30D after 2 hours in PBS at pH 7.4 (150x).

FIGS. HA and HB illustrate a combination of TLR ligands synergistically activate B cells and effectively induces antibody responses. FIG. HA is a bar graph showing expression of CD69 by B cells after 20 hours of stimulation with bone marrow-derived DCs pretreated with TLR ligands in combination (MALP-2+poly(I:C)+CpG). FIG. HB is a bar graph illustrating induction of serum IgG responses against vaccinia proteins A33 and L1 after i.c.r. immunization twice (two-week interval) with recombinant A33+L1 together and the triple TLR ligands formulated in DOTAP. Sera were collected two weeks after the last immunization and measured for antigen-specific IgG by an ELISA assay. **p < 0.01 and ***p < 0.001 indicate difference from each of the other groups.

SEQUENCE LISTING AND NOMENCLATURE

The Sequence Listing is submitted as an ASCII text file, Annex C/St.25 text file, created on August 30, 2010, 2 KB, which is incorporated by reference herein.

The amino acid sequences listed in the accompanying sequence listing are shown using standard three letter code for amino acids, as defined in 37 C.F.R. 1.822. All sequence database accession numbers referenced herein are understood to refer to the version of the sequence identified by that accession number as it was available on the designated date. In the accompanying sequence listing:

SEQ ID NO: 1 is the amino acid sequence of PCLUS3-18IIIB peptide.
SEQ ID NO: 2 is the amino acid sequence of MALP-2S peptide.
SEQ ID NO: 3 is the amino acid sequence of P18-I10 peptide.
SEQ ID NO: 4 is the amino acid sequence of OVA \textsubscript{57-63} peptide.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

1. Terms


Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example." In case of conflict, the present specification, including explanations of terms, will control. In addition, all the materials, methods, and examples are illustrative and not intended to be limiting. All publications, patent applications, patents, GENBANK™ Accession
numbers and other references mentioned herein are incorporated by reference in their entirety.

To facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

**Adjuvant:** A vehicle used to enhance antigenicity; such as a suspension of minerals (alum, aluminum hydroxide, aluminum phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in oil (MF-59, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity (inhibits degradation of antigen and/or causes influx of macrophages). Adjuvants also include immunostimulatory molecules, such as cytokines, costimulatory molecules, and for example, immunostimulatory DNA or RNA molecules.

**Administration:** The introduction of a composition into a subject by a chosen route. For example, if the chosen route is oral delivery, the composition is administered by introducing the composition into the mouth of the subject. Similarly, if the route of administration is to the gastrointestinal tract, the delivery can be anywhere from the duodenum, for example orally, intraesophageal, gastric (for example through a gastric tube) or even duodenally (for example through a duodenal feeding tube).

**Antibody:** A polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds and recognizes an analyte (antigen) such as an HIV envelope polypeptide or an antigenic fragment of an HIV envelope polypeptide. Immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes.

Antibodies exist, for example as intact immunoglobulins and as a number of well characterized fragments produced by digestion with various peptidases. For instance, Fabs, Fvs, and single-chain Fvs (SCFvs) that bind to an HIV envelope polypeptide or fragments of an HIV envelope polypeptide would be HIV-specific binding agents. This includes intact immunoglobulins and the variants and portions of them well known in the art, such as Fab' fragments, F(ab)'2 fragments, single
chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies (such as humanized murine antibodies), heteroconjugate antibodies such as bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., Immunology, 3rd Ed., W.H. Freeman & Co., New York, 1997.

Typically, a naturally occurring immunoglobulin has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE.

Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). In combination, the heavy and the light chain variable regions specifically bind the antigen. Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs." The extent of the framework region and CDRs have been defined (see, Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Department of Health and Human Services, 1991, which is hereby incorporated by reference). The Kabat database is now maintained online. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space.

**Antigen:** A compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions that are injected or absorbed into an animal. Examples include, but are not limited to, peptides, lipids, polysaccharides, and nucleic acids containing antigenic determinants, such as those recognized by an immune cell. In some examples,
antigens include tumor antigens which are antigens expressed on a specific type of
tumor cells.

In some examples, antigens include peptides derived from a pathogen of interest. Exemplary pathogens include bacteria, fungi, viruses and parasites. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. The term is used interchangeably with the term "immunogen." The term "antigen" includes all related antigenic epitopes.

An "antigenic polypeptide" is a polypeptide to which an immune response, such as a T cell response or an antibody response, can be stimulated. An "antigenic polypeptide" includes a polypeptide, such as a polypeptide to an HIV envelope protein (e.g., gp120 or gp41) or a portion thereof that is capable of provoking an immune response in a mammal, such as a mammal with or without a disease, such as an HIV infection. Administration of an antigenic polypeptide that provokes an immune response preferably leads to protective immunity, such as protective immunity against a disease or condition (e.g., protective immunity against HIV).

"Epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. In one embodiment, T cells respond to the epitope when the epitope is presented in conjunction with an MHC molecule. Epitopes can be formed both from contiguous amino acids (linear) or noncontiguous amino acids juxtaposed by tertiary folding of an antigenic polypeptide (conformational). Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 5 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The amino acids are in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and multi-dimensional nuclear magnetic resonance spectroscopy.

The term "antigen" denotes both subunit antigens, (for example, antigens which are separate and discrete from a whole organism with which the antigen is
associated in nature), as well as killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide which expresses an antigen or antigenic determinant in vivo, such as in gene therapy and DNA immunization applications, is also included in the definition of antigen herein.

An "antigen," when referring to a protein or fragment of a protein, includes a protein with modifications, such as deletions, additions and substitutions (generally conservative in nature) to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

**Bacterial pathogen:** A bacteria that causes disease (pathogenic bacteria).

Examples of pathogenic bacteria for which infections that can be treated in accordance with the disclosed methods and compositions include without limitation any one or more of (or any combination of) *Acinetobacter baumanii, Actinobacillus sp., Actinomycetes, Actinomyces sp.* (such as *Actinomyces israelii and Actinomyces naeslundii*), *Aeromonas sp.* (such as *Aeromonas hydrophila, Aeromonas veronii biovar sobria (Aeromonas sobria), and Aeromonas caviae*), *Anaplasma phagocytophilum, Alcaligenes xylosoxidans, Acinetobacter baumanii, Actinobacillus actinomycetemcomitans, Bacillus sp.* (such as *Bacillus anthracis, Bacillus cereus, Bacillus subtilis, Bacillus thuringiensis*, and *Bacillus stea rothermophilus*), *Bacteroides sp.* (such as *Bacteroidesfragilis*), *Bartonella sp.* (such as *Bartonella bacilliformis* and *Bartonella henselae*, *Bifidobacterium sp.*, *Bordetella sp.* (such as *Bordetella pertussis, Bordetella parapertussis*, and *Bordetella bronchiseptica*), *Borrelia sp.* (such as *Borrelia recurrentis, and Borrelia burgdorferi*), *Brucella sp.* (such as *Brucella abortus, Brucella canis, Brucella melintensis and Brucella suis*), *Burkholderia sp.* (such as *Burkholderia pseudomallei* and *Burkholderia cepacia*), *Campylobacter sp.* (such as *Campylobacter jejuni, Campylobacter coli, Campylobacter lari* and *Campylobacterfetus*), *Capnocytophaga sp.*, *Cardiobacterium hominis, Chlamydia trachomatis, Chlamydophila pneumoniae*,
Chlamydophila psittaci, Citrobacter sp. Coxiella burnetii, Corynebacterium sp.
(such as, Corynebacterium diphtheriae, Corynebacterium jeikeium and Corynebacterium), Clostridium sp. (such as Clostridium perfringens, Clostridium difficile, Clostridium botulinum and Clostridium tetani), Eikenella corrodens,
5 Enterobacter sp. (such as Enterobacter aerogenes, Enterobacter agglomerans,
Enterobacter cloacae and Escherichia coli, including opportunistic Escherichia coli,
such as enterotoxigenic E. coli, enteroinvasive E. coli, enteropathogenic E. coli,
enterohemorrhagic E. coli, enteroaggregative E. coli and uropathogenic E. coli)

Enterococcus sp. (such as Enterococcus faecalis and Enterococcus faecium)

Ehrlichia sp. (such as Ehrlichia chafeensis and Ehrlichia canis), Erysipelothrix rhusiopathiae, Eubacterium sp., Francisella tularensis, Fusobacterium nucleatum,
10 Gardnerella vaginalis, Gemella morbillorum, Haemophilus sp. (such as Haemophilus influenzae, Haemophilus ducreyi, Haemophilus aegyptius,
Haemophilus parainfluenzae, Haemophilus haemolyticus and Haemophilus parahaemolyticus, Helicobacter sp. (such as Helicobacter pylori, Helicobacter cinaedi and Helicobacter fennelliae), Kingella kingii, Klebsiella sp. (such as Klebsiella pneumoniae, Klebsiella granulomatis and Klebsiella oxytoca),

(such as Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium intracellular, Mycobacterium avium, Mycobacterium bovis, and Mycobacterium marinum), Mycoplasma sp. (such as Mycoplasma pneumoniae, Mycoplasma hominis, and Mycoplasma genitalium), Nocardia sp. (such as Nocardia asteroides, Nocardia cyriacigeorgica and Nocardia brasiliensis), Neisseria sp. (such as Neisseria gonorrhoeae and Neisseria meningitidis), Pasteurella multocida, Plesiomonas shigelloides. Prevotella sp., Porphyromonas sp., Prevotella melaninogenica,
20 Proteus sp. (such as Proteus vulgaris and Proteus mirabilis), Providencia sp. (such as Providencia alcalifaciens, Providencia rettgeri and Providencia stuartii),
Pseudomonas aeruginosa, Propionibacterium acnes, Rhodococcus equi, Rickettsia sp. (such as Rickettsia rickettsii, Rickettsia akari and Rickettsia prowazekii, Orientia tsutsugamushi (formerly: Rickettsia tsutsugamushi) and Rickettsia typhi),
Rhodococcus sp., Serratia marcescens, Stenotrophomonas maltophilia, Salmonella sp. (such as Salmonella enterica, Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis, Salmonella cholerasuis and Salmonella typhimurium), Serratia sp. (such as Serratia marcesans and Serratia liquefaciens), Shigella sp. (such as Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonneï), Staphylococcus sp. (such as Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hemolyticus, Staphylococcus saprophyticus), Streptococcus sp. (such as Streptococcus pneumoniae (for example chloramphenicol-resistant serotype 4 Streptococcus pneumoniae, spectinomycin-resistant serotype 6B Streptococcus pneumoniae, streptomycin-resistant serotype 9V Streptococcus pneumoniae, erythromycin-resistant serotype 14 Streptococcus pneumoniae, optochin-resistant serotype 14 Streptococcus pneumoniae, rifampicin-resistant serotype 18C Streptococcus pneumoniae, tetracycline-resistant serotype 19F Streptococcus pneumoniae, penicillin-resistant serotype 19F Streptococcus pneumoniae, and trimethoprim-resistant serotype 23F Streptococcus pneumoniae, chloramphenicol-resistant serotype 4 Streptococcus pneumoniae, spectinomycin-resistant serotype 6B Streptococcus pneumoniae, streptomycin-resistant serotype 9V Streptococcus pneumoniae, optochin-resistant serotype 14 Streptococcus pneumoniae, rifampicin-resistant serotype 18C Streptococcus pneumoniae, penicillin-resistant serotype 19F Streptococcus pneumoniae, or trimethoprim-resistant serotype 23F Streptococcus pneumoniae), Streptococcus agalactiae, Streptococcus mutans, Streptococcus pyogenes, Group A streptococci, Streptococcus pyogenes, Group B streptococci, Streptococcus agalactiae, Group C streptococci, Streptococcus anginosus, Streptococcus equisimilis, Group D streptococci, Streptococcus bovis, Group F streptococci, and Streptococcus anginosus Group G streptococci), Spirillum minus, Streptobacillus moniliformi, Treponema sp. (such as Treponema carateum, Treponema pertenue, Treponema pallidum and Treponema endemicum, Tropheryma whippelii, Ureaplasma urealyticum, Veillonella sp., Vibrio sp. (such as Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio alginolyticus, Vibrio mimicus, Vibrio hollisae, Vibrio fluvialis, Vibrio metchnikovii, Vibrio damsela and Vibrio fursnitit), Yersinia sp. (such as Yersinia...
enterocolitica, Yersinia pestis, and Yersinia pseudotuberculosis) and Xanthomonas maltophilia among others.

**Biodegradable** polymer: Polymers that degrade fully (e.g., down to monomeric species) under physiological or endosomal conditions. In an example, the polymers and polymer biodegradation byproducts are biocompatible. Biodegradable polymers are not necessarily hydrolytically degradable and may require enzymatic action to fully degrade.

Biodegradable polymers of the present disclosure with low water solubility or no water solubility (are water-insoluble), include aliphatic polyesters, such as, homopolymers or copolymers synthesized from one or more kinds of α-hydroxycarboxylic acids (such as, glycolic acid, lactic acid, 2-hydroxybutyric acid, valinic acid, leucic acid, and like compounds), hydroxydicarboxylic acids (e.g., malic acid and like compounds), hydroxytricarboxylic acids (e.g., citric acid and like compounds), or mixtures thereof; poly-α-cyanoacrylic esters, such as, poly(methyl α-cyanoacrylate), poly(ethyl α-cyanoacrylate), poly(butyl α-cyanoacrylate), and amino acid polymers, such as, poly(α-benzyl-L-glutamate) or mixtures thereof. The mode of polymerization for these biodegradable polymers can be any, including random, block or graft polymerizations techniques.

Possible biodegradable polymers include aliphatic polyesters, such as, homopolymers or copolymers synthesized from one or more kinds of α-hydroxycarboxylic acids (such as, glycolic acid, lactic acid, 2-hydroxybutyric acid, and like compounds), hydroxydicarboxylic acids (such as, malic acid and like compounds), and hydroxytricarboxylic acids (e.g., citric acid and like compounds), or mixtures thereof. Exemplary aliphatic polyesters include copolymers synthesized from two or more kinds of the α-hydroxycarboxylic acids. Such copolymers can be used as mixtures with, for example, polyethylene glycols (PEGs).

If the α-hydroxycarboxylic acids are chiral compounds, they can D-, L- and D-, or L-configuration. In one example, the ratio of the D-/L-configuration (mol %) is in the range of about 75/25 to about 25/75. For example, a hydroxycarboxylic acid includes a D-/L-configuration (mol %) ratio in the range of about 60/40 to about 30/70, including about 50/50 to about 40/60.
An example of the above mentioned α-hydroxycarboxylic acid polymer is a lactic acid polymer (hereinafter sometimes referred to as "polylactic acid"). The α-hydroxycarboxylic acid copolymer includes copolymers of glycolic acid with the other α-hydroxycarboxylic acids such as lactic acid and 2-hydroxybutyric acid.

Exemplary α-hydroxycarboxylic acid copolymers included lactic acid-glycolic acid copolymer and 2-hydroxybutyric acid-glycolic acid copolymer. Another α-hydroxycarboxylic acid copolymer is a lactic acid-glycolic acid copolymer.

In some examples, the polylactic acid has a weight average molecular weight of about 1,000 to about 100,000, such as a polylactic acid having the weight average molecular weight of about 2,000 to about 80,000. Particularly suitable is a polylactic acid having the weight average molecular weight of about 3,000 to about 60,000. The dispersity (weight average molecular weight/number average molecular weight) of polylactic acid is in the range of about 0.2 to about 4.0, and such as in the range of about 0.5 to about 3.5.

Cancer: A class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood).

The "pathology" of cancer includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc. "Metastatic disease" refers to cancer cells that have left the original tumor site and migrate to other parts of the body, for example via the bloodstream or lymph system. "Mucosal cancer" refers to cancer cells that are present within a mucosal surface, examples include, but are not limited to, colonic or cervical cancer.

Chemotherapy; chemotherapeutic agents: As used herein, any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms, and cancer as well as diseases characterized by hyperplastic growth such as psoriasis. In one
embodiment, a chemotherapeutic agent is an agent of use in treating neoplasms such as solid tumors. In one embodiment, a chemotherapeutic agent is radioactive molecule. One of skill in the art can readily identify a chemotherapeutic agent of use (e.g., see Slapak and Kufe, *Principles of Cancer Therapy*, Chapter 86 in


**Cluster of Differentiation 4** (CD4): A T-cell surface protein that mediates interaction with the MHC class II molecule. CD4 also serves as the primary receptor site for HIV on T-cells during HIV-I infection.  

The known sequence of the CD4 precursor has a hydrophobic signal peptide, an extracellular region of approximately 370 amino acids, a highly hydrophobic stretch with significant identity to the membrane-spanning domain of the class II MHC beta chain, and a highly charged intracellular sequence of 40 residues (Maddon, *Cell* 42:93, 1985).

The term "CD4" includes polypeptide molecules that are derived from CD4 include fragments of CD4, generated either by chemical (for example enzymatic) digestion or genetic engineering means. Such a fragment may be one or more entire CD4 protein domains. The extracellular domain of CD4 consists of four contiguous immunoglobulin-like regions (D1, D2, D3, and D4, see Sakihama *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:6444, 1995; U.S. Patent No. 6,117,655), and amino acids 1 to 183 have been shown to be involved in gpl20 binding. For instance, a binding molecule or binding domain derived from CD4 would comprise a sufficient portion of the CD4 protein to mediate specific and functional interaction between the binding fragment and a native or viral binding site of CD4. One such binding fragment includes both the D1 and D2 extracellular domains of CD4 (D1D2 is also a fragment of soluble CD4 or sCD4 which is comprised of D1 D2 D3 and D4), although smaller fragments may also provide specific and functional CD4-like binding. The gpl20-binding site has been mapped to D1 of CD4.
CD4 polypeptides also include "CD4-derived molecules" which encompasses analogs (non-protein organic molecules), derivatives (chemically functionalized protein molecules obtained starting with the disclosed protein sequences) or mimetics (three-dimensionally similar chemicals) of the native CD4 structure, as well as proteins sequence variants or genetic alleles that maintain the ability to functionally bind to a target molecule.

**Cluster of differentiation 8 (CD8):** A transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). Like the TCR, CD8 binds to a major histocompatibility complex (MHC) molecule, but is specific for the class I MHC protein. There are two isoforms of the protein, alpha and beta, each encoded by a different gene. In humans, both genes are located on chromosome 2 in position 2pl2.

The CD8 co-receptor is predominantly expressed on the surface of cytotoxic T cells, but can also be found on natural killer cells. In one embodiment, a CD8+ T cells is a cytotoxic T lymphocytes. In another embodiment, a CD8 cell is a suppressor T cell. To function, CD8 forms a dimer, consisting of a pair of CD8 chains. The extracellular IgV-like domain of CD8-α interacts with to the \( \alpha_3 \) portion of the Class I MHC molecule. This affinity keeps the T cell receptor of the cytotoxic T cell and the target cell bound closely together during antigen-specific activation.

Coating: As used herein "coating", "coatings", "coated" and "coat" are forms of the same term defining material and process for making a material where a first substance or substrate surface is at least partially covered or associated with a second substance. Both the first and second substance are not required to be different. Thus, a composition composed of a first substance may be "coated" with a second substance via a linking agent that is a third substance. As used herein, the "coating" need not be complete or cover the entire surface of the first substance to be "coated." The "coating" may be complete as well (e.g., approximately covering the entire first substance). There can be multiple coatings and multiple substances within each coating. The coating may vary in thickness or the coating thickness may be substantially uniform. Exemplary compositions of coated particles are disclosed in U.S. Patent No. 6,406,745 (Talton, 2000) which is hereby incorporated by reference in its entirety.
Coatings contemplated in accordance with the present disclosure include, but are not limited to, medicated coatings, drug-eluting coatings, drugs or other compounds, pharmaceutically acceptable carriers and combinations thereof, or any other organic, inorganic or organic/inorganic hybrid materials. In one example, a coating is an anionic copolymer based on methyl acrylate, methyl methacrylate and methacrylic acid, such as EUDRAGIT® (e.g., EUDRAGIT® FS 30 D or EUDRAGIT® RS 100; Evonik Industries, Darmstadt, Germany). In some examples, a coating is a copolymer that is insoluble in acidic media, but dissolves by salt formation above pH 6.5, such as above pH 7.0. In some examples, a coating is a hydrophilic or hydrophobic polymer, such as those containing carboxylic groups, including polyacrylic acid. In certain examples, a coating includes one or more polyanhydride, polyhydroxy acid, or polyesters, such as polylactides, polyglycolides, and blends or copolymers of each. In other examples, a coating includes one or more bioerodible polyhydroxy acids and copolymers thereof, such as polylactic acid, polyglycolic acid, polyhydroxy-butyric acid, polyhydroxy-valeric acid, polycaprolactone, polylactide-co-caprolactone, and polylactide-co-glycolide. In further examples, a coating is a polymer containing labile bonds, such as polyanhydrides and polyorthoesters.

In some examples, a coating is one or more natural polymers, synthetic polymers or combinations thereof. Representative natural polymers include proteins, such as zein, modified zein, casein, gelatin, gluten, serum albumin, or collagen, and polysaccharides such as dextrins, polyhyaluronic acid and alginic acid. Representative synthetic polymers include polyphosphazenes, polyamides, polycarbonates, polycrylamides, polysiloxanes, polyurethanes and copolymers thereof. Celluloses also can be used. As defined herein the term "cellulososes" includes naturally occurring and synthetic cellulososes, such as alkyl cellulososes, cellulose ethers, cellulose esters, hydroxyalkyl cellulososes and nitrocellulososes. Exemplary cellulososes include ethyl cellulose, methyl cellulose, carboxymethyl cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, cellulose triacetate and cellulose sulfate sodium salt.
In some examples, a coating is one or more polymers of acrylic and methacrylic acids or esters and/or copolymers thereof. Representative acrylic and methacrylic acids or esters include polymethyl methacrylate, polyethyl methacrylate, polybutyl methacrylate, polyisobutyl methacrylate, polyhexyl methacrylate, polyisodecyl methacrylate, polyhexyl methacrylate, polymethyl acrylate, polyisopropyl acrylate, polyisobutyl acrylate, and polyoctadecyl acrylate.

In other examples, a coating is one or more polyalkylenes, such as polyethylene and polypropylene; polyaryalkylenes, such as polystyrene; polyalkylene glycols, such as polyethylene glycol; polyalkylene oxides, such as polyethylene oxide; and polyalkylene terephthalates, such as polyethylene terephthalate. In additional examples, a coating is one or more polyvinyl polymers, including polyvinyl alcohols, polyvinyl ethers, polyvinyl esters and polyvinyl halides. In specific examples, a coating includes one or more polyvinyl polymers including polyvinyl acetate, polyvinyl phenol, polyvinylpyrrolidone or a combination thereof.

In some examples, a coating is one or more water soluble or water insoluble polymers. Representative examples of suitable water soluble polymers include polyvinyl alcohol, polyvinylpyrrolidone, methyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose and polyethylene glycol, copolymers of acrylic and methacrylic acid esters, and mixtures thereof. Representative examples of suitable water insoluble polymers include ethylcellulose, cellulose acetate, cellulose propionate (lower, medium or -higher molecular weight), cellulose acetate propionate, cellulose acetate butyrate, cellulose acetate phthalate, cellulose triacetate, polymethyl methacrylate, polyethyl methacrylate, polybutyl methacrylate, polyisobutyl methacrylate, polyhexyl methacrylate, polyisodecyl methacrylate, polylauryl methacrylate, polyethylene methacrylate, polymethyl acrylate, polyisopropyl acrylate, polyisobutyl acrylate, polyoctadecyl acrylate, polyethylene, polyethylene low density, polyethylene high density, polypropylene, polyethylene oxide, polyethylene terephthalate, polyvinyl isobutyl ether, polyvinyl acetate, polyvinyl chloride, polyurethanes, and mixtures thereof. In one example, a water insoluble polymer and a water-soluble polymer are used together, such as in a
mixture. Such mixtures are useful in controlled drug release formulations, wherein the release rate can be controlled by varying the ratio of water-soluble polymer to water insoluble polymer.

Polymers that alter viscosity as a function of temperature or shear or other physical forces can also be used as a coating. Polyoxymethylene polymers and copolymers, such as polyethylene oxide-polypropylene oxide (PEO-PPO), known also as Poloxamer, or polyethylene oxide-poly(butylene oxide) (PEO-PBO) copolymers, and copolymers and blends of these polymers with polymers such as polyalpha-hydroxy acids, including but not limited to lactic, glycolic and hydroxybutyric acids, polycaprolactones, and polyvalerolactones, can be synthesized or commercially obtained (e.g., polyoxymethylene copolymers are commercially available from BASF Wyandotte Corporation (Wyandotte, MI)). For example, these materials are applied as viscous solutions at room temperature or lower which solidify at the higher body temperature. Other materials with similar properties known to one of ordinary skill in the art, including, but not limited to hydroxypropyl cellulose, purified xanthum and guar gums can also be used as coatings.

Other suitable coatings are polymeric lacquer substances based on acrylates and/or methacrylates, such as commercially available EUDRAGIT® polymers (sold by Rohm America, Inc.). Specific EUDRAGIT® polymers can be selected having various permeability and water solubility, which properties can be pH dependent or pH independent. For example, EUDRAGIT® RL and EUDRAGIT® RS are acrylic resins including copolymers of acrylic and methacrylic acid esters with a low content of quaternary ammonium groups, which are present as salts and give rise to the permeability of the lacquer films; EUDRAGIT® RL is freely permeable and EUDRAGIT® RS is slightly permeable, independent of pH. In contrast, the permeability of EUDRAGIT® L is pH dependent. EUDRAGIT® L is an anionic polymer synthesized from methacrylic acid and methacrylic acid methyl ester. It is insoluble in acids and pure water, but becomes increasingly soluble in a neutral to weakly alkaline solution by forming salts with alkalis. In some examples a coating is a matrix, such that particles (for example nanoparticles) are encapsulated by the matrix.
**Colon cancer:** Colorectal cancer, also called large bowel cancer, includes cancerous growths in the colon, rectum and appendix. With 655,000 deaths worldwide per year, it is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. Many colorectal cancers are thought to arise from adenomatous polyps in the colon. These mushroom-like growths are usually benign, but some may develop into cancer over time. The majority of the time, the diagnosis of localized colon cancer is through colonoscopy. Therapy is usually through surgery, which in many cases is followed by chemotherapy. The first symptoms of colon cancer are usually vague, such as bleeding, weight loss, and fatigue (tiredness). Local (bowel) symptoms are rare until the tumor has grown to a large size. Generally, the nearer the tumor is to the anus, the more bowel symptoms there will be.

**Degenerate variant and conservative variant:** A polynucleotide encoding a polypeptide or an antibody that includes a sequence that is degenerate as a result of the genetic code. Examples include a polynucleotide encoding an HIV envelope polypeptide or an antibody that binds an HIV envelope polypeptide, such as gp41 or gp120, which includes a sequence that is degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included as long as the amino acid sequence of the specific polypeptide (such as a gp41 or gp120 polypeptide) or antibody that binds the specific polypeptide encoded by the nucleotide sequence is unchanged. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified within a protein encoding sequence, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations," which are one species of conservative variations. Each nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation.

One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a
functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

Furthermore, one of ordinary skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (for instance less than 5%, in some embodiments less than 1%) in an encoded sequence are conservative variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid.

Conservative amino acid substitutions providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Not all residue positions within a protein will tolerate an otherwise "conservative" substitution. For instance, if an amino acid residue is essential for a function of the protein, even an otherwise conservative substitution may disrupt that activity.

**Epitope:** An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, such that they elicit a specific immune response. An antibody binds a particular antigenic epitope, such as an epitope of an HIV polypeptide, such as a gp41 polypeptide or a gpl20 polypeptide.

**Expression:** Translation of a nucleic acid into a protein. Proteins may be expressed and remain intracellular, become a component of the cell surface membrane, or be secreted into the extracellular matrix or medium.

**Expression Control Sequences:** Nucleic acid sequences that regulate the expression of a heterologous nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when
the expression control sequences control and regulate the transcription and, as
appropriate, translation of the nucleic acid sequence. Thus expression control
sequences can include appropriate promoters, enhancers, transcription terminators, a
start codon (ATG) in front of a protein-encoding gene, splicing signal for introns,
maintenance of the correct reading frame of that gene to permit proper translation of
mRNA, and stop codons. The term "control sequences" is intended to include, at a
minimum, components whose presence can influence expression, and can also
include additional components whose presence is advantageous, for example, leader
sequences and fusion partner sequences. Expression control sequences can include a
promoter.

A promoter is a minimal sequence sufficient to direct transcription. Also
included are those promoter elements which are sufficient to render promoter-
dependent gene expression controllable for cell-type specific, tissue-specific, or
inducible by external signals or agents; such elements may be located in the 5' or 3'
regions of the gene. Both constitutive and inducible promoters are included (see for
example, Bitter et al, Methods in Enzymology 153:516-544, 1987). For example,
when cloning in bacterial systems, inducible promoters such as pL of bacteriophage
lambda, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. In one
embodiment, when cloning in mammalian cell systems, promoters derived from the
genome of mammalian cells (such as metallothionein promoter) or from mammalian
viruses (such as the retrovirus long terminal repeat; the adenovirus late promoter; the
vaccinia virus 7.5K promoter) can be used. Promoters produced by recombinant
DNA or synthetic techniques may also be used to provide for transcription of the
nucleic acid sequences.

A polynucleotide can be inserted into an expression vector that contains a
promoter sequence which facilitates the efficient transcription of the inserted genetic
sequence of the host. The expression vector typically contains an origin of
replication, a promoter, as well as specific nucleic acid sequences that allow
phenotypic selection of the transformed cells.

**Fungal pathogen:** A fungus that causes disease. Examples of fungal
pathogens for which infections that can be treated in accordance with the disclosed
methods and compositions include without limitation any one or more of (or any

combination of Trichophyton rubrum, T. mentagrophytes, Epidermophyton floccosum, Microsporum canis, Pityrosporum orbiculare (Malassezia furfur), Candida sp. (such as Candida albicans), Aspergillus sp. (such as Aspergillus fumigatus, Aspergillus flavus and Aspergillus clavatus), Cryptococcus sp. (such as Cryptococcus neoformans, Cryptococcus gattii, Cryptococcus laurentii and Cryptococcus albidus), Histoplasma sp. (such as Histoplasma capsulatum), Pneumocystis sp. (such as Pneumocystis jiroveci), and Stachybotrys (such as Stachybotrys chartarum).

Glycoprotein **41 (gp41)**: An HIV-I envelope glycoprotein that mediates receptor binding and HIV entry into a cell. Gp41 includes a MPR and a transmembrane spanning domain. Gp41 is immunogenic and induces a variety of neutralizing antibodies, such as neutralizing antibodies directed to 2F5, 4E10 and Z13. These three gp41 neutralizing antibodies recognize the MPR of the HIV-I gp41 glycoprotein.

Glycoprotein **120 (gpl20)**: An envelope protein from Human Immunodeficiency Virus (HIV). The envelope protein is initially synthesized as a longer precursor protein of 845-870 amino acids in size, designated gpl60. Gpl60 forms a homotrimer and undergoes glycosylation within the Golgi apparatus. It is then cleaved by a cellular protease into gpl20 and gp41. Gp41 contains a transmembrane domain and remains in a trimeric configuration; it interacts with gpl20 in a non-covalent manner. Gpl20 contains most of the external, surface-exposed, domains of the envelope glycoprotein complex, and it is gpl20 which binds both to the cellular CD4 receptor and to the cellular chemokine receptors (such as CCR5).

The mature gpl20 wildtype polypeptides have about 500 amino acids in the primary sequence. Gp120 is heavily N-glycosylated giving rise to an apparent molecular weight of 120 kD. The polypeptide is comprised of five conserved regions (C1-C5) and five regions of high variability (V1-V5). Exemplary sequence of wt gpl60 polypeptides are shown on GENBANK, for example accession numbers AAB05604 and AAD12142

The gpl20 core has a unique molecular structure, which comprises two domains: an "inner" domain (which faces gp41) and an "outer" domain (which is
mostly exposed on the surface of the oligomeric envelope glycoprotein complex). The two gpl20 domains are separated by a "bridging sheet" that is not part of either domain. The gpl20 core comprises 25 beta strands, 5 alpha helices, and 10 defined loop segments.

Gpl20 polypeptides also include "gpl20-derived molecules" which encompasses analogs (non-protein organic molecules), derivatives (chemically functionalized protein molecules obtained starting with the disclosed protein sequences) or mimetics (three-dimensionally similar chemicals) of the native gpl20 structure, as well as proteins sequence variants (such as mutants), genetic alleles, fusions proteins of gpl20, or combinations thereof.


**Host cells:** Cells in which a vector can be propagated and its DNA expressed. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used.

**Immune response:** A response of a cell of the immune system, such as a B cell, T cell, or monocyte, to a stimulus. In one embodiment, the response is specific for a particular antigen (an "antigen-specific response"). In one embodiment, an immune response is a T cell response, such as a CD4+ response or a CD8+ response. In another embodiment, the response is a B cell response, and results in the production of specific antibodies.

**Immunogen:** An agent (such as an antigen) capable of stimulating a specific immune response against a target, such as a pathogen or tumor.

**Immunogenic peptide:** A peptide which comprises an allele-specific motif or other sequence such that the peptide will bind an MHC molecule and induce a
cytotoxic T lymphocyte ("CTL") response, or a B cell response (for example antibody production) against the antigen from which the immunogenic peptide is derived.

In one embodiment, immunogenic peptides are identified using sequence motifs or other methods, such as neural net or polynomial determinations known in the art. Typically, algorithms are used to determine the "binding threshold" of peptides to select those with scores that give them a high probability of binding at a certain affinity and will be immunogenic. The algorithms are based either on the effects on MHC binding of a particular amino acid at a particular position, the effects on antibody binding of a particular amino acid at a particular position, or the effects on binding of a particular substitution in a motif-containing peptide. Within the context of an immunogenic peptide, a "conserved residue" is one which appears in a significantly higher frequency than would be expected by random distribution at a particular position in a peptide. In one embodiment, a conserved residue is one where the MHC structure may provide a contact point with the immunogenic peptide. In one specific non-limiting example, an immunogenic polypeptide includes a region of gpl20, or a fragment thereof.

**Immunogenic composition:** A composition comprising an immunogenic peptide that induces a measurable CTL or other T cell response against virus expressing the immunogenic peptide, or induces a measurable B cell response (such as production of antibodies) against the immunogenic peptide. In one example an "immunogenic composition" is composition comprising an HIV envelope polypeptide that induces a measurable CTL response against a virus expressing an HIV envelope protein, or induces a measurable B cell response (such as production of antibodies) against an HIV envelope polypeptide. It further refers to isolated nucleic acids encoding an immunogenic peptide, such as a nucleic acid that can be used to express the HIV envelope polypeptide (and thus be used to elicit an immune response against this polypeptide).

For *in vitro* use, an immunogenic composition may consist of the isolated protein, peptide epitope, or nucleic acid encoding the protein, or peptide epitope. For *in vivo* use, the immunogenic composition will typically comprise the protein or immunogenic peptide in pharmaceutically acceptable carriers, and/or other agents.
Any particular peptide, such as an HIV envelope polypeptide, or nucleic acid encoding the polypeptide, can be readily tested for its ability to induce a T cell or B cell response by art-recognized assays. Immunogenic compositions can include adjuvants, which are well known to one of skill in the art.

**Immunologically reactive conditions:** Includes reference to conditions which allow an antibody raised against a particular epitope to bind to that epitope to a detectably greater degree than, and/or to the substantial exclusion of, binding to substantially all other epitopes. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols or those conditions encountered *in vivo*. The immunologically reactive conditions employed in the methods are "physiological conditions" which include reference to conditions (such as temperature, osmolality, pH) that are typical inside a living mammal or a mammalian cell. While it is recognized that some organs are subject to extreme conditions, the intra-organismal and intracellular environment is normally about pH 7 (such as from pH 6.0 to pH 8.0, more typically pH 6.5 to 7.5), contains water as the predominant solvent, and exists at a temperature above 0 °C and below 50 °C. Osmolality is within the range that is supportive of cell viability and proliferation.

**Immunostimulatory agent:** An adjunct agent different from an immunogen that is administered with an immunogen to enhance an immune response against the immunogen. Examples include one or more TLR ligands, cytokines or a combination thereof. In some examples, one or more TLR ligands include TLR ligands provided in Table 1 (e.g., MALP2, poly(I:C), CpG 2343 or combination thereof). Exemplary cytokines include, but are not limited to, IL-15, IL-12 or GM-CSF.

**Immunotherapy:** A method of evoking an immune response against a virus or cancer based on their production of target antigens. Immunotherapy based on cell-mediated immune responses involves generating a cell-mediated response to cells that produce particular antigenic determinants, while immunotherapy based on humoral immune responses involves generating specific antibodies to virus that produce particular antigenic determinants.
Immunostimulatory **CpG motifs**: Immunostimulatory nucleic acid sequences that trigger macrophages, monocytes and lymphocytes to produce a variety of pro-inflammatory cytokines and chemokines. CpG motifs represent a type of Toll like receptor TLR ligand. Examples of immunostimulatory oligonucleotides that include CpG motifs are found for example in U.S. Patent No. 6,194,388; U.S. Patent No. 6,207,646; U.S. Patent No. 6,214,806; U.S. Patent No. 6,218,371; U.S. Patent No. 6,239,116; U.S. Patent No. 6,339,068; U.S. Patent No. 6,406,705; and U.S. Patent No. 6,429,199.

**Inhibiting or treating a disease**: Inhibiting the full development of a disease or condition, for example, in a subject who is at risk for a disease such as an STD including acquired immune deficiency syndrome (AIDS), an AIDS related conditions, an HIV-I infection, or combinations thereof. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. The term "ameliorating," with reference to a disease or pathological condition, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of metastases, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular disease. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology.

**Isolated**: An "isolated" biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, such as, other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides, and proteins prepared by
recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Macrophage-activating lipopeptide-2 (MALP-2): A diacylated lipopeptide isolated from Mycoplasma fermentans. MALP-2 is a Toll-like receptor (TLR) agonist and is believed to bind the complex of TLR 2 and TLR 6. MALP-2S or S-[2,3-bispalmitoyloxy-(2#)-propyl]-cysteinyl-GNNDESNSFKEK (SEQ ID NO: 2) is the synthetic analogue of MALP-2. As used herein, MALP-2 refers to the natural product isolated from Mycoplasma fermentans as well as synthetic analogues, such as MALP-2S.

Nanoparticle: A microscopic particle whose size is measured in nanometers (nm). It is defined as a particle that does not have a dimension > 1000 nm, such as having a size between about 10 and about 1000 nm, for example, between about 10 and about 100 nm, between 100 and about 500 nm, or between about 500 and about 1000 nm. Nanoparticles are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size, but at the nano-scale this is often not the case. Size-dependent properties are observed such as quantum confinement in semiconductor particles, surface plasmon resonance in some metal particles and superparamagnetism in magnetic materials. Semi-solid and soft nanoparticles have been manufactured. A prototype nanoparticle of semi-solid nature is the liposome.

At the small end of the size range, nanoparticles are often referred to as clusters. Metal, dielectric, and semiconductor nanoparticles have been formed, as well as hybrid structures (e.g., core-shell nanoparticles). Nanospheres, nanorods, and nanocups are just a few of the shapes that have been grown. Semiconductor quantum dots and nanocrystals are types of nanoparticles. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents.

Nanoparticle characterization is necessary to establish understanding and control of nanoparticle synthesis and applications. Characterization is done by using a variety of different techniques, mainly drawn from materials science. Common techniques are electron microscopy [transmission or scanning, abbreviated TEM or SEM respectively], atomic force microscopy [AFM], dynamic light scattering
[DLS], x-ray photoelectron spectroscopy [XPS], powder x-ray diffractometry [XRD], and Fourier transform infrared spectroscopy [FTIR].

Naturally **Occurring Amino Acids:** L-isomers of the naturally occurring amino acids. The naturally occurring amino acids are glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine, gamma-carboxyglutamic acid, arginine, ornithine and lysine. Unless specifically indicated, all amino acids referred to in this application are in the L-form. "Synthetic amino acids" refers to amino acids that are not naturally found in proteins. Examples of synthetic amino acids used herein, include racemic mixtures of selenocysteine and selenomethionine. In addition, unnatural amino acids include the D or L forms of nor-leucine, para-nitrophenylalanine, homophenylalanine, para-fluorophenylalanine, 3-aminobenzylpropionic acid, homoarginine, and D-phenylalanine. The term "positively charged amino acid" refers to any naturally occurring or synthetic amino acid having a positively charged side chain under normal physiological conditions. Examples of positively charged naturally occurring amino acids are arginine, lysine and histidine. The term "negatively charged amino acid" refers to any naturally occurring or synthetic amino acid having a negatively charged side chain under normal physiological conditions. Examples of negatively charged naturally occurring amino acids are aspartic acid and glutamic acid. The term "hydrophobic amino acid" refers to any amino acid having an uncharged, nonpolar side chain that is relatively insoluble in water. Examples of naturally occurring hydrophobic amino acids are alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The term "hydrophilic amino acid" refers to any amino acid having an uncharged, polar side chain that is relatively soluble in water. Examples of naturally occurring hydrophilic amino acids are serine, threonine, tyrosine, asparagine, glutamine, and cysteine.

**Nucleic acid:** A polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Thus, the term includes nucleotide polymers in which the nucleotides and
the linkages between them include non-naturally occurring synthetic analogs, such
as, for example and without limitation, phosphorothioates, phosphoramidates,
methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides,
peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be
synthesized, for example, using an automated DNA synthesizer. The term
"oligonucleotide" typically refers to short polynucleotides, generally no greater than
about 50 nucleotides. It will be understood that when a nucleotide sequence is
represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA
sequence (i.e., A, U, G, C) in which "U" replaces "T."

"Nucleotide" includes, but is not limited to, a monomer that includes a base
linked to a sugar, such as a pyrimidine, purine or synthetic analogs thereof, or a base
linked to an amino acid, as in a peptide nucleic acid (PNA). A nucleotide is one
monomer in a polynucleotide. A nucleotide sequence refers to the sequence of bases
in a polynucleotide. An HIV envelope polynucleotide is a nucleic acid encoding an
HIV envelope polypeptide.

Conventional notation is used herein to describe nucleotide sequences: the
left-hand end of a single-stranded nucleotide sequence is the 5'-end; the left-hand
direction of a double-stranded nucleotide sequence is referred to as the 5'-direction.
The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is
referred to as the transcription direction. The DNA strand having the same sequence
as an mRNA is referred to as the "coding strand;" sequences on the DNA strand
having the same sequence as an mRNA transcribed from that DNA and which are
located 5' to the 5'-end of the RNA transcript are referred to as "upstream
sequences;" sequences on the DNA strand having the same sequence as the RNA
and which are 3' to the 3' end of the coding RNA transcript are referred to as
"downstream sequences."

"cDNA" refers to a DNA that is complementary or identical to an mRNA, in
either single stranded or double stranded form.

"Encoding" refers to the inherent property of specific sequences of
nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as
templates for synthesis of other polymers and macromolecules in biological
processes having either a defined sequence of nucleotides (for example, rRNA,
tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Recombinant nucleic acid" refers to a nucleic acid having nucleotide sequences that are not naturally joined together. This includes nucleic acid vectors comprising an amplified or assembled nucleic acid which can be used to transform a suitable host cell. A host cell that comprises the recombinant nucleic acid is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, such as a "recombinant polypeptide." A recombinant nucleic acid may serve a non-coding function (such as a promoter, origin of replication, ribosome-binding site, etc.) as well.

A first sequence is an "antisense" with respect to a second sequence if a polynucleotide whose sequence is the first sequence specifically hybridizes with a polynucleotide whose sequence is the second sequence.

Terms used to describe sequence relationships between two or more nucleotide sequences or amino acid sequences include "reference sequence," "selected from," "comparison window," "identical," "percentage of sequence identity," "substantially identical," "complementary," and "substantially complementary."

For sequence comparison of nucleic acid sequences and amino acids sequences, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are
designated. Default program parameters are used. Methods of alignment of
sequences for comparison are well known in the art. Optimal alignment of
sequences for comparison can be conducted, for example, by the local homology
algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970, by the
85:2444, 1988, by computerized implementations of these algorithms (GAP,
BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package,
Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment
and visual inspection (see for example, *Current Protocols in Molecular Biology*
(Ausubel *et al.*, eds 1995 supplement)).

One example of a useful algorithm is PILEUP. PILEUP uses a
simplification of the progressive alignment method of Feng & Doolittle, *J. Mol.
Evol.* 35:351-360, 1987. The method used is similar to the method described by
is compared to other test sequences to determine the percent sequence identity
relationship using the following parameters: default gap weight (3.00), default gap
length weight (0.10), and weighted end gaps. PILEUP can be obtained from the
GCG sequence analysis software package, such as version 7.0 (Devereaux *et al.*,

Another example of algorithms that are suitable for determining percent
sequence identity and sequence similarity are the BLAST and the BLAST 2.0
algorithm, which are described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990
performing BLAST analyses is publicly available through the National Center for
Biotechnology Information on the worldwide web at ncbi.nlm.nih.gov. The
BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of
11, alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of
both strands. The BLASTP program (for amino acid sequences) uses as defaults a
word length (W) of 3, and expectation (E) of 10, and the BLOSUM62 scoring
Another indicia of sequence similarity between two nucleic acids is the ability to hybridize. The more similar are the sequences of the two nucleic acids, the more stringent the conditions at which they will hybridize. The stringency of hybridization conditions are sequence-dependent and are different under different environmental parameters. Thus, hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na\(^+\) and/or Mg\(^{2+}\) concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times also influence stringency. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T\(_m\)) for the specific sequence at a defined ionic strength and pH. The T\(_m\) is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Conditions for nucleic acid hybridization and calculation of stringencies can be found, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001; Tijssen, Hybridization With Nucleic Acid Probes, Part I: Theory and Nucleic Acid Preparation, Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Ltd., NY, NY, 1993 and Ausubel et al. Short Protocols in Molecular Biology, 4th ed., John Wiley & Sons, Inc., 1999.

"Stringent conditions" encompass conditions under which hybridization will only occur if there is less than 25% mismatch between the hybridization molecule and the target sequence. "Stringent conditions" may be broken down into particular levels of stringency for more precise definition. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 25% sequence mismatch will not hybridize; conditions of "medium stringency" are those under which molecules with more than 15% mismatch will not hybridize, and conditions of "high stringency" are those under which sequences with more than 10% mismatch will not hybridize. Conditions of "very high stringency" are those under which sequences with more than 6% mismatch will not hybridize. In contrast nucleic acids that hybridize under "low stringency conditions include those with
much less sequence identity, or with sequence identity over only short subsequences of the nucleic acid. For example, a nucleic acid construct can include a polynucleotide sequence that hybridizes under high stringency or very high stringency, or even higher stringency conditions to a polynucleotide sequence that encodes SEQ ID NO: 1.

**Parasite:** An organism that lives inside humans or other organisms acting as hosts (for the parasite). Parasites are dependent on their hosts for at least part of their life cycle. Parasites are harmful to humans because they consume needed food, eat away body tissues and cells, and eliminate toxic waste, which makes people sick.

Exemplary parasites that can be treated with the compositions and methods disclosed herein include Malaria (Plasmodium falciparum, P. vivax, P. malariae), Schistosomes, Trypanosomes, Leishmania, Filarial nematodes, Trichomoniasis, Sarcosporidiasis, Taenia (T. saginata, T. solium), Leishmania, Toxoplasma gondii, Trichinelosis (Trichinella spiralis) or Coccidiosis (Eimeria species).

**Peptide:** Any compound composed of amino acids, amino acid analogs, chemically bound together. Peptide as used herein includes oligomers of amino acids, amino acid analog, or small and large peptides, including polypeptides or proteins encompasses any chain of amino acids, regardless of length or post-translational modification (such as glycosylation or phosphorylation). "Peptide" applies to amino acid polymers to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer as well as in which one or more amino acid residue is a non-natural amino acid, for example a artificial chemical mimic of a corresponding naturally occurring amino acid. A "residue" refers to an amino acid or amino acid mimic incorporated in a polypeptide by an amide bond or amide bond mimic. A peptide has an amino terminal (N-terminal) end and a carboxy terminal (C-terminal) end. "Peptide" is used interchangeably with polypeptide or protein, and is used interchangeably herein to refer to a polymer of amino acid residues.

Amino acids generally are chemically bound together via amide linkages (CONH). Additionally, amino acids may be bound together by other chemical bonds. For example, linkages for amino acids or amino acid analogs can include CII₂NII₂, -(IhS-)···1₂-CII₂CII₂···CII-(cis and trans), ···OCI₁₂···.

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**Peptides may be modified by a variety of chemical techniques to produce derivatives** having essentially the same activity as the unmodified proteins, and optionally having other desirable properties. For example, carboxylic acid groups of the protein, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a Ci-Ci ester, or converted to an amide of formula NR1R2 wherein R1 and R2 are each independently H or C1-C6 alkyl, or combined to form a heterocyclic ring, such as a 5- or 6-membered ring. Amino groups of the protein, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to Ci-Ci alkyl or dialkyl amino or further converted to an amide.

Hydroxyl groups of the protein side chains may be converted to Ci-Ci alkoxy or to a Ci-Ci ester using well-recognized techniques. Phenyl and phenolic rings of the protein side chains may be substituted with one or more halogen atoms, such as fluorine, chlorine, bromine or iodine, or with Ci-Ci alkyl, Ci-Ci alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the protein side chains can be extended to homologous C2-C4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetalamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the proteins to select and provide conformational constraints to the structure that result in enhanced stability.
In one embodiment, the polypeptide is an HIV envelope protein, such as a gp41 or gpl20 polypeptide. A "residue" refers to an amino acid or amino acid mimetic incorporated in a polypeptide by an amide bond or amide bond mimetic. A polypeptide has an amino terminal (N-terminal) end and a carboxy terminal (C-terminal) end.

**Pharmaceutical agent:** A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject or a cell. An "anti-viral agent" or "anti-viral drug" is an agent that specifically inhibits a virus from replicating or infecting cells. Similarly, an "anti-retroviral agent" is an agent that specifically inhibits a retrovirus from replicating or infecting cells.


In general, the nature of the carrier or excipient will depend on the particular mode of administration being employed. For instance, for solid compositions (such as pill or tablet), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

**Poly(lactic-co-glycolic acid) or PLGA:** A biodegradable copolymer. PLGA is synthesized by means of random ring-opening co-polymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. Common catalysts used in the preparation of this polymer include tin(II) 2-ethylhexanoate, tin(II) alkoxides, or aluminum isopropoxide. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding a linear, aliphatic polyester as a product.
Depending on the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained: these are usually identified in regard to the monomers' ratio used (e.g., PLGA 75:25 identifies a copolymer whose composition is 75% lactic acid and 25% glycolic acid). PLGAs are amorphous rather than crystalline and show a glass transition temperature in the range of 40-60 °C.

Unlike the homopolymers of lactic acid (polylactide) and glycolic acid (polyglycolide) which show poor solubilities, PLGA can be dissolved by a wide range of common solvents, including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate.

In some examples, the compositional ratio (lactic acid/glycolic acid, mol %) in the lactic acid-glycolic acid copolymer is about 100/0 (homopolymer) to about 0/100, such as about 90/10 to about 85/15, about 75/25 to 25/75, or about 60/40 to about 40/60. The weight average molecular weight of the lactic acid-glycolic acid copolymer is about 1,000 to about 20,000, such as about 4,000 to about 15,000. The dispersity (weight average molecular weight/number average molecular weight) of the lactic acid-glycolic acid copolymer is about 0.2 to about 4.0, such as about 0.5 to about 3.5. In certain examples, a mixture of a lactic acid-glycolic acid copolymer has a compositional ratio of lactic acid/glycolic acid (mol %) of about 50/50 and the weight average molecular weight of about 6,000. In other examples, a lactic acid-glycolic acid copolymer has a compositional ratio of lactic acid/glycolic acid (mol %) of about 50/50 and the weight average molecular weight of about 4,000. The suitable weight ratio of the mixture is about 25/75 to about 75/25.

PLGA degrades by hydrolysis of its ester linkages in the presence of water. It has been shown that the time required for degradation of PLGA is related to the monomers' ratio used in production: the higher the content of glycolide units, the lower the time required for degradation. In some examples, a composition includes a target immunogen encapsulated by PLGA.

Polyinosinic-polycytidylic acid (Poly I:C): Is a Toll like receptor (TLR) agonist. Poly I:C is believed to interact with Toll-like receptor (TLR) 3, which is expressed in the intracellular compartments of B-cells and dendritic cells.

Sexually Transmitted Disease or Infection (SID): An infection transmitted between subjects by means of sexual contact, including vaginal
intercourse, oral sex, and anal sex. This term as used herein includes a subject that may be infected, and may potentially infect others, without showing signs of disease.

A sexually transmitted disease is also known as a venereal disease (VD). Exemplary SIDs include can be bacterial (such as Chancroid (*Haemophilus ducreyi*),

Donovanosis (*Granuloma inguinale* or *Calymmatobacterium granulomatis*), Gonorrhea (*Neisseria gonorrhoeae*), Lymphogranuloma venereum (LGV) (*Chlamydia trachomatis* serotypes L₁, L₂, L₃. See Chlamydia), Non-gonococcal urethritis (NGU) (*Ureaplasma urealyticum* or *Mycoplasma hominis*), Staphylococcal infection (*Staphylococcus aureus*, MRSA) and Syphilis (*Treponema pallidum*),

fungal (such as *Trichophyton rubrum*), parasitic (such as *Phthirius pubis* or *Sarcoptes scabiei*) or viral (such as adenoviruses, Hepatitis (e.g., Hepatitis A, Hepatitis B, or Hepatitis E), Herpes simplex virus (1, T), HIV/ AIDS, human T-lymphotropic virus (HTLV 1 or T), human papilloma virus, molluscum contagiosum virus, cytomegalovirus, Epstein-Barr virus, SARS, or Kaposi’s sarcoma-associated herpес virus).

**Subject:** Living multi-cellular vertebrate organisms, a category that includes both human and veterinary subjects, including human and non-human mammals.

**T Cell:** A white blood cell critical to the immune response. T cells include, but are not limited to, CD4⁺ T cells and CD8⁺ T cells.

**Therapeutic agent:** Used in a generic sense, it includes treating agents, prophylactic agents, and replacement agents.

Therapeutically **effective amount:** An amount of a composition that alone, or together with an additional therapeutic agent(s) induces the desired response (e.g., inhibition of HIV infection or replication).

A therapeutically effective amount of a composition including the active agent can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the therapeutically effective amount can depend on the subject being treated, the severity and type of the condition being treated, and the manner of administration. For example, a therapeutically effective amount of such agent can vary from about 1 mg to 1 gram per 70 kg body weight if administered orally.
Toll-like receptors (TLR): A class of proteins that play a role in the innate immune system. They are single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs which activates immune cell responses.

TLRs are a type of pattern recognition receptor (PRR) and recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). TLRs together with the Interleukin-1 receptors form a receptor superfamily, known as the "Interleukin-1 Receptor/Toll-Like Receptor Superfamily"; all members of this family have in common a so-called TIR (Toll-IL-1 receptor) domain.

Three subgroups of TIR domains exist. Proteins with subgroup 1 TIR domains are receptors for interleukins that are produced by macrophages, monocytes and dendritic cells and all have extracellular Immunoglobulin (Ig) domains. Proteins with subgroup 2 TIR domains are classical TLRs, and bind directly or indirectly to molecules of microbial origin. A third subgroup of proteins containing TIR domains consists of adaptor proteins that are exclusively cytosolic and mediate signaling from proteins of subgroups 1 and 2.

TLRs are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having a different ligand specificity. TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS, which requires MD-2. CD14 and LPS Binding Protein (LBP) are known to facilitate the presentation of LPS to MD-2.

When activated, TLRs recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. Four adapter molecules are known to be involved in signaling. These proteins are known as MyD88, Tirap (also called Mal), Trif, and Tram. The adapters activate other molecules within the cell, including certain protein kinases (IRAK1, IRAK4, TBK1, and IKKi) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response. In all, thousands of genes are activated by TLR signaling,
and collectively, the TLRs constitutes one of the most pleiotropic yet tightly regulated gateways for gene modulation.

Toll-like receptors recognize molecules that are constantly associated with threats (e.g., pathogen or cell stress) and are highly specific to these threats (e.g., cannot be mistaken for self molecules). These molecules are known as **TLR ligands**. Exemplary TLR ligands are provided in the Table 1 below.

### Table 1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand(s)</th>
<th>Location</th>
<th>Cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 1</td>
<td>multiple triacyl lipopeptides</td>
<td>Bacteria</td>
<td>cell surface monocytes/macrophages a subset of dendritic cells B lymphocytes</td>
</tr>
<tr>
<td></td>
<td>multiple glycolipids</td>
<td>Bacteria</td>
<td>cell surface monocytes/macrophages Myeloid dendritic cells Mast cells</td>
</tr>
<tr>
<td></td>
<td>multiple lipopeptides</td>
<td>Bacteria</td>
<td>cell surface monocytes/macrophages Myeloid dendritic cells Mast cells</td>
</tr>
<tr>
<td></td>
<td>multiple lipoproteins</td>
<td>Bacteria</td>
<td>cell surface monocytes/macrophages Myeloid dendritic cells Mast cells</td>
</tr>
<tr>
<td></td>
<td>lipoteichoic acid</td>
<td>Bacteria</td>
<td>cell surface monocytes/macrophages Myeloid dendritic cells Mast cells</td>
</tr>
<tr>
<td></td>
<td>HSP70</td>
<td>Host cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>zymosan</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td>TLR 2</td>
<td>Numerous others, such as MALP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR 3</td>
<td>double-stranded RNA, poly I:C</td>
<td>viruses</td>
<td>cell compartment Dendritic cells B lymphocytes</td>
</tr>
<tr>
<td>TLR 4</td>
<td>lipopolysaccharide</td>
<td>Gram-negative bacteria</td>
<td>cell surface monocytes/macrophages Myeloid dendritic cells Mast cells Intestinal epithelium</td>
</tr>
<tr>
<td></td>
<td>several heat shock proteins</td>
<td>Bacteria and host cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fibrinogen</td>
<td>host cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>heparan sulfate fragments</td>
<td>host cells</td>
<td></td>
</tr>
</tbody>
</table>
Following activation by ligands of microbial origin, several reactions are possible. Immune cells can produce signaling factors called cytokines which trigger inflammation. In the case of a bacterial factor, the pathogen might be phagocytosed and digested, and its antigens presented to CD4+ T cells. In the case of a viral
factor, the infected cell may shut off its protein synthesis and may undergo
programmed cell death (apoptosis). Immune cells that have detected a virus may
also release anti-viral factors such as interferons.

In one particular example, a TLR ligand is a molecule that activates a TLR
located on the mucosal surface of the lower gastrointestinal tract, for example a TLR
expressed on a dendritic cell. For instance, a TLR ligand is a molecule that activates
a TLR expressed on a dendritic cell resulting in augmentation of immunity by
enhancing the T cell quality (especially avidity). In some examples, a Toll-like
receptor (TLR) ligand is MALP-2 or an analogue of MALP-2, such as MALP-2S.

In some examples, a Toll-like receptor (TLR) ligand is poly I:C. In some examples,
a Toll-like receptor (TLR) ligand is an immunostimulatory oligonucleotide (such as
those including a CpG motif). Some combinations that are identified here as
synergistically active as vaccine adjuvants include MALP-2 and Poly I:C, Poly LC
and CpG, or a triple combination of all three, MALP2, Poly LC and CpG (Zhu et al,
J. Clin. Invest. 120(2): 607-616, 2010, which is hereby incorporated by reference in
its entirety). Some combinations that are not synergistic or even additive include
2008, which is hereby incorporated by reference in its entirety).

**Tumor antigen:** A tumor antigen is an antigen produced by tumor cells that
can stimulate tumor-specific T-cell immune responses. Exemplary tumor antigens
include, but are not limited to, RAGE-I, tyrosinase, MAGE-I, MAGE-2, NY-ESO-
1, Melan-A/MART-1, glycoprotein (gp) 75, gp100, beta-catenin, preferentially
expressed antigen of melanoma (PRAME), MUM-I, Wilms tumor (WT)-I,
carcinoembryonic antigen (CEA), and PR-I. Additional tumor antigens are known
in the art (for example see Novellino et al, Cancer Immunol. Immunother.
54(3): 187-207, 2005) and are described below. Tumor antigens are also referred to
as "cancer antigens."

**Tumor, cancer, neoplasia or** malignancy: The result of abnormal and
uncontrolled growth of cells. Neoplasia, malignancy, cancer and tumor are often
used interchangeably and refer to abnormal growth of a tissue or cells that results
from excessive cell division. Hematological cancers are cancers of the blood or
bone marrow. Examples of hematological tumors include leukemias, including
acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas and are named for the type of cells that form them. Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma and retinoblastoma).

**Vaccine:** A pharmaceutical composition that elicits a prophylactic or therapeutic immune response in a subject. In some cases, the immune response is a protective immune response. Typically, a vaccine elicits an antigen-specific immune response to an antigen of a pathogen, for example, a bacterial or viral pathogen, or to a cellular constituent correlated with a pathological condition, such as cancer. A vaccine may include a polynucleotide, a peptide or polypeptide, a virus, a bacterium, a cell or one or more cellular constituents. In some cases, the virus, bacteria or cell may be inactivated or attenuated to prevent or reduce the
likelihood of infection, while maintaining the immunogenicity of the vaccine constituent. The immunogenic material may include live-attenuated or killed microorganisms (such as bacteria or viruses), or antigenic proteins, peptides or DNA derived from them. In some cases, the vaccine is a subunit vaccine, which is an immunizing agent that has been treated to remove traces of nucleic acid (such as viral nucleic acid) so that only protein subunits remain. The subunits have less risk of causing adverse reactions. The vaccine can also be a live vaccine, which is a vaccine prepared from living attenuated organisms or from viruses that have been attenuated but can still replicate in the cells of the host organism.

The immunogenic material for a cancer vaccine may include, for example, a protein or peptide expressed by a tumor or cancer cell. Vaccines may elicit both prophylactic (preventative) and therapeutic responses.

**Vector:** A vaccine vector is a virus, bacterium, or other microbe, or a nucleic acid, used to deliver an antigen or a gene for an antigen, as part of a vaccine. A nucleic acid vector is a nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. Recombinant DNA vectors are vectors having recombinant DNA. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements known in the art. Viral vectors are recombinant DNA vectors having at least some nucleic acid sequences derived from one or more viruses.

**Virus:** A microscopic infectious organism that reproduces inside living cells. A virus consists essentially of a core of nucleic acid surrounded by a protein coat, and has the ability to replicate only inside a living cell. "Viral replication" is the production of additional virus by the occurrence of at least one viral life cycle. A virus may subvert the host cells' normal functions, causing the cell to behave in a manner determined by the virus. For example, a viral infection may result in a cell producing a cytokine, or responding to a cytokine, when the uninfected cell does not normally do so. In some examples, a virus is a pathogen.

"Retroviruses" are RNA viruses wherein the viral genome is RNA. When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA
of infected cells. The integrated DNA intermediate is referred to as a provirus. The
term "lentivirus" is used in its conventional sense to describe a genus of viruses
containing reverse transcriptase. The lentiviruses include the "immunodeficiency
viruses" which include human immunodeficiency virus (HIV) type 1 and type 2
(HIV-I and HIV-II), simian immunodeficiency virus (SIV), and feline
immunodeficiency virus (FIV).

HIV-I is a retrovirus that causes immunosuppression in humans (HIV
disease), and leads to a disease complex known as AIDS. "HIV disease" refers to a
well-recognized constellation of signs and symptoms (including the development of
opportunistic infections) in persons who are infected by an HIV virus, as determined
by antibody or western blot studies. Laboratory findings associated with this disease
are a progressive decline in T cells.

Specific examples of viral pathogens for which an infection can be treated
in accordance with the disclosed methods include, without limitation; Arenaviruses
(such as Guanarito virus, Lassa virus, Junin virus, Machupo virus and Sabia),
Arteriviruses, Roniviruses, Astroviruses, Bunyaviruses (such as Crimean-Congo
hemorrhagic fever virus and Hantavirus), Barnaviruses, Birnaviruses, Bornaviruses
(such as Borna disease virus), Bromoviruses, Caliciviruses, Chrysovirus,
Coronaviruses (such as Coronavirus and SARS), Cystoviruses, Closteroviruses,
Comoviruses, Dicistroviruses, Flaviruses (such as Yellow fever virus, West Nile
virus, Hepatitis C virus, and Dengue fever virus), Filoviruses (such as Ebola virus
and Marburg virus), Flexiviruses, Hepeviruses (such as Hepatitis E virus), human
adenoviruses (such as human adenovirus A-F), human astroviruses, human BK
polyomaviruses, human bocaviruses, human coronavirus (such as a human
coronavirus HKU1, NL63, and OC43), human enteroviruses (such as human
enterovirus A-D), human erythrovirus V9, human foamy viruses, human
herpesviruses (such as human herpesvirus 1 (herpes simplex virus type 1), human
herpesvirus 2 (herpes simplex virus type 2), human herpesvirus 3 (Varicella zoster
virus), human herpesvirus 4 type 1 (Epstein-Barr virus type 1), human herpesvirus 4
type 2 (Epstein-Barr virus type 2), human herpesvirus 5 strain AD169, human
herpesvirus 5 strain Merlin Strain, human herpesvirus 6A, human herpesvirus 6B,
human herpesvirus 7, human herpesvirus 8 type M, human herpesvirus 8 type P and
Human Cytomegalovirus), human immunodeficiency viruses (HIV) (such as HIV 1 and HIV 2), human metapneumoviruses, human papillomaviruses (such as human papillomavirus-1, human papillomavirus-18, human papillomavirus-2, human papillomavirus-54, human papillomavirus-61, human papillomavirus-cand90, human papillomavirus RTRX7, human papillomavirus type 10, human papillomavirus type 101, human papillomavirus type 103, human papillomavirus type 107, human papillomavirus type 16, human papillomavirus type 24, human papillomavirus type 26, human papillomavirus type 32, human papillomavirus type 34, human papillomavirus type 4, human papillomavirus type 41, human papillomavirus type 48, human papillomavirus type 49, human papillomavirus type 5, human papillomavirus type 50, human papillomavirus type 53, human papillomavirus type 60, human papillomavirus type 63, human papillomavirus type 6b, human papillomavirus type 7, human papillomavirus type 71, human papillomavirus type 9, human papillomavirus type 92, and human papillomavirus type 96), human parainfluenza viruses (such as human parainfluenza virus 1-3), human parechoviruses, human paroviruses (such as human parovirus 4 and human parovirus B19), human respiratory syncytial viruses, human rhinoviruses (such as human rhinovirus A and human rhinovirus B), human spumaretroviruses, human T-lymphotropic viruses (such as human T-lymphotropic virus 1 and human T-lymphotropic virus 2), Human polyoma viruses, Hypoviruses, Leviviruses, Luteoviruses, Lymphocytic choriomeningitis viruses (LCM), Marnaviruses, Narnaviruses, Nidovirales, Nodaviruses, Orthomyxoviruses (such as Influenza viruses), Partitiviruses, Paramyxoviruses (such as Measles virus and Mumps virus), Picornaviruses (such as Poliovirus, the common cold virus, and Hepatitis A virus), Potyviruses, Poxviruses (such as Variola and Cowpox), Sequiviruses, Reoviruses (such as Rotavirus), Rhabdoviruses (such as Rabies virus), Rhabdoviruses (such as Vesicular stomatitis virus, Tetraviruses, Togaviruses (such as Rubella virus and Ross River virus), Tombusviruses, Totiviruses, Tymoviruses, and Noroviruses among others.

Weight average molecular weight and number average molecular weight: The polystyrene equivalent average molecular weight and number average molecular weight of a sample as determined by gel permeation chromatography
(GPC) using 9 polystyrene standards having the weight average molecular weights of 120,000, 52,000, 22,000, 9,200, 5,050, 2,950, 1,050, 580 and 162. These determinations can be made using GPC Column Millenium Stryragel HE 4E (Waters), an HP 1047A refractive index detector (Hewlett Packard), and THF as the mobile phase.

II. Description of Several Embodiments

A. Introduction

Immunization in the large intestine, by the intrarectal route, has been a preferred method for the protection against viral infections transmitted through the rectal mucosa. Moreover, induction of gut immune responses by vaccination at the large intestine also confers vaginal protection against virus challenge. While this immunization route is believed superior to that induced at a distant site, such as a parenteral or intranasal route, intrarectal administration is considered impractical and invasive. On the other hand, oral vaccine delivery is desirable because it would increase compliance as more subjects would be willing to obtain treatment. However, oral vaccination has been shown to be ineffective for this purpose because antigens are enzymatically or chemically degraded and absorbed during the passage through the stomach and small intestine prior to reaching the lower gastrointestinal tract. How to best deliver vaccines to the large intestine for the induction of desired mucosal immunity is a significant challenge. Thus, the need exists for compositions and methods of treatment for immunization in the lower gastrointestinal tract such as the large intestine using oral delivery.

To deliver vaccines orally specifically to reach the large intestine, it is desirable to keep them intact, avoid uptake in the upper part of the GI tract, e.g., the stomach and small intestine, before entering the target site. FIG. 1A provides a schematic illustration of an exemplary oral delivery system. With reference to FIG. 1A, an exemplary oral delivery system 100 includes a polymer core 120 encapsulating a therapeutically effective amount of a target immunogen 130 and a therapeutically effective amount one or more immunostimulatory agents 140 to form encapsulated particles 150. The particle 150 is a matrix composed of a mixture of polymer core 120 surrounding and suspending a therapeutically effective amount of
target immunogen 130 and a therapeutically effective amount of one or more immunostimulatory agents 140. Target immunogens can include an immunogen that stimulates protective immunity against a target, such as multiple identical immunogens and multiple identical immunostimulatory agents contained within the surrounding polymer matrix 120. Multiple encapsulated particles 150 are in turn suspended within, coated by and encapsulated within a pH-sensitive matrix 160 to form an immunogenic composition capable of eliciting an immune response in the intestine.

FIG. 1B provides an illustration of an exemplary method of oral vaccine delivery to the large intestine. To circumvent the digestive destruction of orally administered vaccines to target the large intestine, a micro/nanoparticle/vaccine complex system has been developed for oral delivery of immunogens and other immunogenic compositions to large intestine, with minimal perturbation in upper gastrointestinal regions. In some examples PLGA nanoparticles encapsulate vaccine components thereby forming particles in a size range at which efficient particle uptake by the gut can be attained once they are in the lumen and in contact with the intestinal mucosal surface. PLGA nanoparticles are pre-coated with an excipient that is pH sensitive (such as EUDRAGIT®) to form larger, micrometer-sized particles to prevent particles from degradation and uptake in the upper gastrointestinal segments. The exterior coating of a pH sensitive excipient, such as EUDRAGIT®, is designed to dissolve and be able to release contents (e.g., PLGA nanoparticles) at high pH (>6.5, for example greater than 7.0), such as the relatively less acidic conditions measured in the large intestine. Thus, the disclosed oral vaccination system allows site-specific delivery of a vaccine contained within the PGLA nanoparticles to the large intestine. This method allows the transfer of antigens and adjuvants in the form of peptide or protein (or nucleic acid) while protected by excipients. As disclosed herein the oral vaccination system also enhances the immune response induced by the vaccine by invoking combinatorial targeting of multiple TLRs to improve host defense.

TLRs recognize viruses, bacteria, fungi, and protozoa by specific ligation to a certain range of components of these microbes. The mucosal surface of the lower gastrointestinal tract expresses various TLRs. These TLRs expressed in the lower
gastrointestinal tract play a role in the induction of innate and adaptive immunity against these microorganisms. Dendritic cells are found to express almost all TLRs, and are able to produce inflammatory cytokines, such as IL-12, synergistically when encountering certain combinations of TLR ligands. As disclosed herein, selective combinations of TLR ligands can have substantial impacts on DCs and the downstream T-cell response. Significantly, it was determined that a triple combination of TLR ligands boosts dendritic cells to produce IL-15, and thus augments immunity by enhancing the T cell quality (especially avidity), in contrast to the quantity compared to using double combinations of TLR ligands. Using an HIV model, it is demonstrated that immunization with the triple TLR ligands using the disclosed micro/nanoparticle/vaccine complex system provided significant protection against intrarectal challenge with SIVmac251.

**B. Oral Delivery System**

Disclosed herein is an oral delivery system that allows site-specific delivery of a vaccine to the large intestine as well as an enhanced immune response induced by the vaccine once delivered to the target site. In one embodiment, the system includes immunogenic compositions with one or more particles, such as nanoparticles, having a core including a bioactive agent (such as a target immunogen) and an immunostimulatory agent (such as a TLR ligand) encapsulated by a controlled-release polymer and a pH-sensitive copolymer matrix (also known as a coating) disposed about the encapsulated core, for example a pH-sensitive copolymer matrix that becomes soluble in high pH, for example pH greater than about pH 6.5, such as greater than about pH 6.6, greater than about pH 6.7, greater than about pH 6.8, greater than about pH 6.9, greater than about pH 7.0, greater than about pH 7.1, greater than about pH 7.2, greater than about pH 7.3, greater than about pH 7.4, or even greater than about pH 7.5. Such compositions can be ingested by a subject, for example by oral administration. The core can include nanoparticles, having a size between about 10 and about 1000 nm, for example, between about 10 and about 100 nm, between 100 and about 500 nm, or between about 500 and about 1000 nm. Following coating, the nanoparticles are encapsulated in microparticles having a size between about 1 and about 100 micrometers, for example, between about 1 and about 10 micrometers, between
about 10 and about 50 micrometers, or between about 50 and about 100 micrometers. The particles upon reaching the large intestine gradually release the bioactive agent (e.g., a target immunogen) at a rate determined by the decomposition rate of the core. In some examples, sustained-release profiles of PLGA nanoparticles provide 6 to 12 hour release in vitro. In other examples, faster release occurs such as less than about 6 hours, for example less than about 5 hours, less than about 4 hours, less than about 3 hours, less than about 2 hours or even less than about 1 hour.

It is contemplated that the disclosed oral delivery system can be used to control STDs. It is also envisioned that this novel vaccine design can be useful in the development of vaccines against other emerging diseases, including infectious diseases as well as the prevention of cancer.

i. **Bioactive Agents (such as Immunogens and Immunostimulatory agents)**

The bioactive agent is an agent that effects the desired therapeutic effects, such as stimulation of an immune response (such as a specific immune response) against a target pathogen of interest (such as a viral or bacterial pathogen of interest that causes a disease in a host, such as a sexually transmitted disease, for example a disease that infects or is transmitted through the lower intestinal tract of the subject, for example a pathogen that infects the rectal mucosa). In particular examples, the bioactive agent is an immunogen, such as an immunogen derived from the pathogenic organisms listed in the foregoing listing of terms, which provides a specific protective immune response against the pathogen of interest. Illustrative examples of bioactive agents that can be included in the disclosed immunogenic compositions include peptide, protein, polysaccharide, glycolipid, glycoprotein, or fragments thereof. For example, the target immunogen can be a subunit, fragment or element of, or derived from, an antigen. For example, the immunogen is an antigenic polypeptide of a pathogenic organism, such as a viral, fungal, parasitic or bacterial organism that produces undesirable symptoms in a subject following exposure. The immunogen may for example be an attenuated virus, bacteria, fungus and/or parasite. Mixtures thereof are also contemplated by the present disclosure.
some examples, the immunogen includes only a part of a microorganism, such as viruses, bacteria and fungi. For example such a part may be a viral capsid or a fragment thereof. Alternatively, the bioactive agent (e.g., immunogen) includes one or more molecules, which have been derived from viruses, bacteria and fungi, such as for example polypeptides, polysaccharides, glycolipids, glycoproteins, or nucleic acid sequences. In some embodiments, a bioactive agent (e.g., immunogen) includes molecules such as for example polypeptides, polysaccharides, glycolipids, glycoproteins, or nucleic acid sequences, which include only fragments of viral, bacterial, fungi and parasite derived polypeptides, polysaccharides, glycolipids, glycoproteins, or nucleic acid sequences. Such molecules can include more than one fragment.

In certain examples, the bioactive agent (e.g., immunogen) is an antigen derived from a pathogen. Exemplary pathogens include viruses, bacteria, fungi or parasites. A non-limiting, and far from exhaustive list of viruses, bacteria, fungi and parasites from which antigen can be derived is provided in the listing of terms provided above.

In some particular examples, the bioactive agent (e.g., immunogen) is an antigen derived from a pathogen involved in a sexually transmitted disease, such as *Haemophilus ducreyi*, *Granuloma inguinale*, *Calymmatobacterium granulomatis*, *Neisseria gonorrhoeae*, *Lymphogranuloma venereum*, *Chlamydia trachomatis* (serotypes L1, L2, L3), *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Staphylococcus aureus*, *Treponema pallidum*, *Trichophyton rubrum*, *Phthirius pubis*, *Sarcoptes scabiei*, an adenovirus, Hepatitis (e.g., Hepatitis A, Hepatitis B, or Hepatitis E), Herpes simplex virus (1, 2), HIV/AIDS, human T-lymphotropic virus (HTLV 1 or 2), human papilloma virus, molluscum contagiosum virus, cytomegalovirus, Epstein-Barr virus, SARS, or Kaposi's sarcoma-associated herpes virus.

In some examples, a bioactive agent (e.g., immunogen) is any antigen that is used as a vaccine for a single disease ("single antigen") or two or more diseases simultaneously ("mixed antigen"). The mixed antigen may be a mixture of two or more antigens, or an antigen that has antigenicities for two or more diseases simultaneously, e.g., a recombinant protein. As an antigen, there may be used an
entire organism, e.g., a viral or bacterial whole cell, or a part of the organism, e.g., a certain protein having an antigenicity.

In a further example, a bioactive agent includes more than one different polypeptide and/or peptide, such as 2, for example 3, such as 4, for example 5, such as 6, for example 7, such as 8, for example 9, such as 10, for example more than 10 different polypeptides. In other examples, the immunogen comprises or essentially consists of an organism, preferably a microorganism or part of an organism, preferably a microorganism and accordingly the antigen comprises a very large number of different polypeptides, such as more than 100, for example more than 500, such as more than 1000, for example more than 2500.

It is also contained within the present disclosure that the antigen may essentially consist of or consist of one or more polypeptides and/or peptides. In a certain example, a bioactive agent is at least one HIV envelope CTL polypeptide, such as a gp41 or gpl20 peptide. In a particular embodiment, a bioactive agent is a PCLUS3-18IIIB peptide. For example, the bioactive agent has an amino acid sequence as provided by SEQ ID NO: 1.

In some examples, the disclosed immunogenic compositions include an immunogen and an immunostimulatory agent, to enhance the immune response induced by the bioactive agent. Exemplary immunostimulatory agents are known to those of skill in the art and include TLR ligands. In certain examples, one or more TLR ligands are included within the immunogenic composition, such as at least two, at least three, at least four, at least five TLR ligands, for example 2, 3, 4, 5, 6, 7, 8, 9 or 10 TLR ligands. Exemplary ligands are provided in Table 1. In a particular example, the composition includes TLR ligands, MALP2, poly(I:C) and CpG.

h. Encapsulation Materials

In some examples, a disclosed immunogenic composition includes an encapsulation material to encapsulate or surround the bioactive agent and/or immunostimulatory agent (such as a TLR ligand). For example, the bioactive active agent (e.g., immunogen) and/or immunostimulatory agent are encapsulated with one or more encapsulating pharmaceuticals to form particles in a size range at which efficient particle uptake can be reached in the lumen of the large intestine. Materials
for use in encapsulating pharmaceuticals for use with the disclosure can be biodegradable or non-biodegradable. A variety of biodegradable polymers are well known to those skilled in the art. Exemplary synthetic polymers suitable for use with this disclosure include but are not limited to poly(arylates), poly(anhydrides), poly(hydroxy acids), polyesters, poly(ortho esters), polycarbonates, poly(propylene fumarates), poly(caprolactones), polyamides, polyphosphazenes, polyamino acids, polyethers, polyacetals, polylactides, polyhydroxyalkanoates, polyglycolides, polyketal, polyesteramides, poly(dioxanones), polyhydroxybutyrates, polyhydroxyvalyrates, polycarbonates, polyorthocarbonates, polyvinyl pyrrolidone, biodegradable polycyanoacrylates, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(methyl vinyl ether), poly(ethylene imine), poly(acyrlie acid), poly(maleic anhydride), biodegradable polyurethanes and polysaccharides. U.S. patents that describe the use of polyanhydrides for controlled delivery of substances include U.S. Pat. No. 4,857,311, U.S. Pat. No. 4,888,176 and U.S. Pat. No. 4,789,724.

Naturally-occurring polymers, such as polysaccharides and proteins, may also be employed. Exemplary polysaccharides include alginate, starches, dextrans, celluloses, chitin, chitosan, hyaluronic acid and its derivatives; exemplary proteins include collagen, albumin, and gelatin. Polysaccharides such as starches, dextrans, and celluloses may be unmodified or may be modified physically or chemically to affect one or more of their properties such as their characteristics in the hydrated state, their solubility, or their half-life in vivo.

In other embodiments, the polymer includes polyhydroxy acids such as polylactic acid (PLA), polyglycolic acid (PGA), their copolymers poly(lactic-co-glycolic acid) (PLGA), and mixtures of any of these. These polymers are among the synthetic polymers approved for human clinical use as surgical suture materials and in controlled release devices. They are degraded by hydrolysis to products that can be metabolized and excreted. Furthermore, copolymerization of PLA and PGA offers the advantage of a large spectrum of degradation rates from hours to several months, such as less than about 6 hours, for example less than about 5 hours, less than about 4 hours, less than about 3 hours, less than about 2 hours or even less than
about 1 hour, by varying the copolymer ratio of glycolic acid to lactic acid, which is more hydrophobic and less crystalline than PGA and degrades at a slower rate.

Non-biodegradable polymers may also be employed for use with the disclosure. Exemplary non-biodegradable, yet biocompatible polymers include polystyrene, polyesters, non-biodegradable polyurethanes, polyureas, poly(vinyl alcohol), poly amides, poly(tetrafluoroethylene), poly(ethylene vinyl acetate), polypropylene, polyacrylate, non-biodegradable polycyanoacrylates, non-biodegradable polyurethanes, polymethacrylate, poly(methyl methacrylate), polyethylene, polypyrrole, polyanilines, polythiophene, and poly(ethylene oxide).

Any of the above polymers may be functionalized with a poly(alkylene glycol), for example, poly(ethylene glycol) (PEG) or poly(propylene glycol) (PPG), or may have a particular terminal functional group, e.g., poly(lactic acid) modified to have a terminal carboxyl group. Exemplary PEGylated polymers include but are not limited to PEGylated poly(lactic acid), PEGylated poly(lactic-co-glycolic acid), PEGylated poly(caprolactone), PEGylated poly(ortho esters), PEGylated polylysine, and PEGylated poly(ethylene imine). Poly(alkylene glycols) are known to increase the bioavailability of many pharmacologically useful compounds, partly by increasing the gastrointestinal stability of derivatized compounds. Poly(alkylene glycols) chains may be as short as about 100 Daltons or have a molecular weight of about 1000, about 3000, about 5000, about 7000 Daltons, or more. The poly(alkylene glycol) chain may also be modified to have a charged endgroup or other group selected to engage in a particular interaction with the coating material. For example, carboxylated PEG will engage in electrostatic interactions with positively charged coating materials such as chitosan.

Co-polymers, mixtures, and adducts of any of the above modified and unmodified polymers may also be employed. For example, amphiphilic block co-polymers having hydrophobic regions and anionic or otherwise hydrophilic regions may be employed. Block co-polymers having regions that engage in different types of non-covalent or covalent interactions may also be employed. For example, a block co-polymer may have one block that is optimized to interact with an active agent being encapsulated and another block optimized to interact with the bioadhesive coating. Alternatively or in addition, polymers may be chemically
modified to have particular functional groups. For example, polymers may be functionalized with hydroxyl, amine, carboxy, maleimide, thiol, N-hydroxy-succinimide (NHS) esters, or azide groups. These groups may be used to render the polymer hydrophilic or to achieve particular interactions with coating materials as described below.

One skilled in the art will recognize that the molecular weight and the degree of cross-linking may be adjusted to control the decomposition rate of the polymer and thus the release rate of the pharmaceutical. Methods of controlling molecular weight and cross-linking to adjust release rates are well known to those skilled in the art.

A variety of methods of making particles in which active agents are encapsulated are well known to those skilled in the art. For example, a double emulsion technique may be used to combine a polymer and active agent in particles. Alternatively, particles may be prepared by spray-drying.

iii. Carrier Materials

In some examples, the disclosed immunogenic composition includes one or more carrier or coating materials to protect the disclosed immunogenic compositions from degradation prior to reaching the target site (e.g., the large intestine). Example compositions of particle carriers are available in U.S. Patent Nos. 6,406,745 (Talton, 2000) and U.S. Patent Application No. 20050175707, each of which is incorporated herein by reference in its entirety.

Positively charged biocompatible materials are suitable for coating or protecting particles for use with this disclosure. For example, chitosan, poly(L-lysine), and poly(ethylene imines) are suitable for coating particles for use with the disclosure. Lectins may also be used to coat particles. Lectins may particularly target M cells in Peyer’s patches in the intestine, enhancing the affinity of the particles for the intestinal wall. Lectins are produced by a wide variety of plants; one skilled in the art will recognize that not all lectins are appropriate for use in pharmaceutical compositions. A wide variety of lectins are available from Sigma-Aldrich, which also provides information on the toxicity and mutagenicity of commercially available lectins. One skilled in the art will recognize that lectins that
are found in commonly eaten foods are more likely to be suitable for use with embodiments of the disclosure.

In some example, negatively charged materials are employed as carrier/coating materials. Exemplary bioadhesive materials include, without limitation, lecithin, polycarboxylic acids, poly(acrylic acids), polysaccharides, monosaccharides, oligosaccharides, oligopeptides, polypeptides, and co-polymers of two or more mucoadhesive materials. Alternatively or in addition, mucoadhesive or non-mucoadhesive polymers may be modified with mucoadhesive materials. For example, sugars may be covalently linked to polyacrylates. Polymers having regions adapted to bind the coating to the core material and regions adapted to be mucoadhesive may also be employed. For example, a block co-polymer of a polycation and a hydrogen bond donor can be used to coat a core containing a polymer that acts as a hydrogen bond receptor. Additional bioadhesive molecules that may be used with the disclosure include but are not limited to hydrophilic and amphiphilic polymers, hydrogels, and the polymers disclosed in U.S. Pat. Nos. 6,217,908, 6,297,337; 6,514,535; and 6,284,235.

One skilled in the art will recognize that excessive cross-linking of the carrier/coating material may hinder release of the active agent from the core of the particle. The skilled artisan will also recognize that the effect of cross-linking may be easily tested by measuring the release of an active agent or a labeled analog from particles coated with materials having different degrees of cross-linking.

In another embodiment, the particles are provided with a double coating. For example, the particles may include a targeting agent that helps direct the particles to a specific tissue once they enter the blood stream. Exemplary targeting agents include nucleic acid aptamers, growth factors, hormones, cytokines, interleukins, antibodies, integrins, fibronectin receptors, p-glycoprotein receptors, and cell binding sequences such as RGD. Nucleic acid aptamers selective for a particular target may be known from the literature or may be identified using any method known to those skilled in the art, for example, the methods disclosed in U.S. Pat. Nos. 5,270,163, 5,475,096, and 6,114,120. Aptamers for certain tissues may also be obtained commercially, for example, from Archemix Corp. These targeting agents may be attached to the surface of the particle or may be attached to the polymer
itself before the particles are formed. The particles are then encapsulated with a negatively charged material, e.g., a negatively charged polymer. Exemplary polymers include carboxymethylcellulose, polyacrylic acid, polymethacrylic acid, polystyrenesulfonate, and polymers including carboxylate, sulfonate, sulfate, phosphate, or nitrate groups. A positively charged carrier/coating material is then coated over the negatively charged material, such as a copolymer based on methyl acrylate, methyl methacrylate and methacrylic acid, including EUDRAGIT® (Evonik Industries, Darmstadt, Germany).

The particles produced using the techniques described herein may be sufficiently small to traverse the intestinal mucosa or the alveolar wall. Enhanced uptake may be achieved for larger particles by the use of mucosal coatings, as described herein. The size of the particle may be optimized for stability and increased uptake.

After the particle crosses the intestinal wall into the bloodstream, the environmental pH increases from about 2-3 to about 7.4. Depending on the pKa of the negative coating, it may become neutrally charged, reducing its affinity for the positively charged mucoadhesive coating. As a result, the mucoadhesive coating becomes dislodged from the particle. The negatively charged carrier material or coating may also be biodegradable, for example, through hydrolysis or enzymatic mechanisms. In this embodiment, whether the pKa of the negatively charged carrier material or coating is such that it will become neutrally charged after entering the bloodstream, the degradation of the material/coating will dislodge the outer mucoadhesive material/coating from the particle. In any of these embodiments, the two coatings protect both the agent being delivered and the targeting agent from degradation in the digestive system while allowing the targeting agent to be exposed at the surface of the particles after they enter the bloodstream.

In one certain example, a pH sensitive microparticle carrier is used to protect the disclosed PLGA nanoparticles disclosed herein from degradation prior to reaching the target site (e.g., the large intestine). For example, the carrier is an anionic copolymer based on methyl acrylate, methyl methacrylate and methacrylic acid, such as EUDRAGIT® (Evonik Industries, Darmstadt, Germany). In some examples, a carrier/coating is a copolymer that is insoluble in acidic media, but
dissolves by salt formation above about pH 6.5, such as about pH 6.6, about pH 6.7, about pH 6.8, about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, about pH 7.4, or about pH 7.5. In some examples, a carrier/coating is a copolymer that is insoluble in acidic media, but dissolves by salt formation above pH 7.0. In a particular example, a carrier/coating is EUDRAGIT® FS 30 D or EUDRAGIT® RS 100 (Evonik Industries, Darmstadt, Germany). For example, EUDRAGIT® coats the PLGA nanoparticles to protect PLGA from degradation prior to reaching the target site (e.g., the large intestine).

iv. Formation of a Coated Particle

Carriers/Coatings may be immobilized on the particles using a variety of chemical interactions. For example, positively charged carriers/coatings such as chitosan will form electrostatic bonds with negatively charged PLA or PLGA. This interaction prevents the carrier/coating from being stripped off the particle as it passes into the bloodstream. Likewise, negatively charged carriers/coatings may be employed with positively charged cores.

The electrostatic interaction allows for easy fabrication of the particles and facilitates release of the active agent. Layer-by-layer deposition techniques may be used to coat the particles. For example, particles may be suspended in a solution containing the coating material, which then simply adsorbs onto the surface of the particles. The coating is not a thick or tight layer but rather allows the active agent to diffuse from the polymer core into the bloodstream. In addition, where enzymatic action is needed to decompose the core, the carrier/coating allows enzymes to diffuse from the blood into the particle. Even though the carrier/coating can remain intact as the active agent is released, it is itself susceptible to decomposition, and the particle can be fully metabolized.

In addition to electrostatic interactions, other non-covalent interactions may also be used to immobilize a coating. Exemplary non-covalent interactions include but are not limited to the following:

1) Affinity Interactions: For example, biotin may be attached to the surface of the controlled release polymer core and streptavidin may be attached to the carrier/coating material; or conversely, biotin may be attached to the carrier/coating
material and the streptavidin may be attached to the surface of the controlled release polymer core. The biotin group and streptavidin are typically attached to the controlled release polymer system or to the coating via a linker, such as an alkylene linker or a polyether linker. Biotin and streptavidin bind via affinity interactions, thereby retaining the carrier/coating on the controlled release polymer core.

2) Metal Coordination: For example, a polyhistidine may be attached to or included within the carrier/coating material, and a nitrilotriacetic acid can be attached to the surface of the controlled release polymer core. A metal, such as Ni^{2+}, will chelate the polyhistidine and the nitrilotriacetic acid, thereby binding the carrier/coating to the controlled release polymer core.

3) Physical Adsorption: For example, a hydrophobic tail, such as polymethacrylate or an alkyl group having at least about 10 carbons, may be attached to the carrier/coating material. The hydrophobic tail will adsorb onto the surface of a hydrophobic controlled release polymer, such as a polyorthoester, polysebacic anhydride, unmodified poly(lactic acid), or polycaprolactone, thereby binding the carrier/coating to the controlled release polymer core.

4) Host-Guest Interactions: For example, a macrocyclic host, such as cucurbituril or cyclodextrin, may be attached to the controlled release polymer or the surface of the controlled release polymer core and a guest group, such as an alkyl group, a polyethylene glycol, or a diaminoalkyl group, may be attached to the carrier/coating material; or conversely, the host group may be attached to the carrier/coating material and the guest group may be included in the controlled release polymer core. In one embodiment, the host and/or the guest molecule may be attached to the carrier/coating material or the controlled release polymer system via a linker, such as an alkylene linker or a polyether linker.

5) Hydrogen Bonding Interactions: For example, an oligonucleotide having a particular sequence may be attached to the surface of the controlled release polymer core, and an essentially complementary sequence may be attached to the carrier/coating material. The carrier/coating material will then bind to the controlled release polymer core via complementary base pairing with the oligonucleotide attached to the controlled release polymer system. Two oligonucleotides are essentially complimentary if about 80% of the nucleic acid bases on one
oligonucleotide form hydrogen bonds via an oligonucleotide base pairing system, such as Watson-Crick base pairing, reverse Watson-Crick base pairing, Hoogsten base pairing, etc., with a base on the second oligonucleotide. Typically, it is desirable for an oligonucleotide sequence attached to the controlled release polymer system to form at least about 6 complementary base pairs with a complementary oligonucleotide attached to the nucleic acid ligand. For example, a poly(cytosine) tag may be attached to the controlled release polymer core and a poly(guanine) tag may be attached to the coating material. Indeed, it is not necessary to only surface treat the controlled release polymer; the entire polymer may be so modified. Some of the poly-C tags will end up on the surface of the core, and others will remain in the interior portions of the particle. In another embodiment, sugars may be used as a mucoadhesive coating. The hydroxyl groups on sugars such as glucose and galactose will hydrogen bond with polar moieties on polymers such as polyvinyl alcohol. Sugar dimers or oligomers may be used as well.

The core and the carrier/coating may also be linked via covalent interactions. For example, PLGA may be modified with a carboxylate group and employed as a core material. Chitosan or another aminated coating material can be coupled to the core using a coupling reagent such as EDC or DCC. Alternatively, PLGA may be modified to have an activated NHS ester which can then be reacted with an amine group on the coating material. Either carrier/coating or core materials may be modified to include reactive groups such as hydroxyl, amine, carboxyl, maleimide, thiol, NHS ester, azide, or alkyne. Standard coupling reactions may then be used to couple the modified material to a second material having a complementary group (e.g., a carboxyl modified core coupled to an aminated carrier/coating material).

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the encapsulated particle is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, (b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, (c) humectants such as glycerol, (d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain
silicates, and sodium carbonate, (e) solution retarding agents such as paraffin, (f) absorption accelerators such as quaternary ammonium compounds, (g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, (h) absorbents such as kaolin and bentonite clay, and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art.

C. Methods of Treatment

In some examples, the disclosed immunogenic compositions are administered to a subject in an immunologically effective dose in a suitable form to prevent and/or treat infectious diseases. The particular dosage depends upon the age, weight and medical condition of the subject to be treated, as well as on the method of administration. Suitable doses can be readily determined by those of skill in the art.

It will be appreciated that the exact dosage is chosen by the individual physician in view of the patient to be treated. In general, dosage and administration are adjusted to provide an effective amount of the desired active agent to the subject being treated. As will be appreciated by those of ordinary skill in the art, the effective amount of bioactive agent may vary depending on such factors as the desired biological endpoint, the active agent and/or immunostimulatory agents to be delivered, the target tissue, the route of administration, etc. For example, the severity of the disease state; age, weight and gender of the patient being treated; diet, time and frequency of administration; drug combinations; reaction sensitivities; and tolerance/response to therapy.
The disclosed immunogenic compositions are preferably compounded with a carrier in dosage unit form for ease of administration and uniformity of dosage. The expression "dosage unit form" as used herein refers to a physically discrete unit of conjugate appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the compositions of the immunogenic compositions will be decided by the attending physician within the scope of sound medical judgment. For any particle composition, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, pigs, or non-human primates. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. Therapeutic efficacy and toxicity of particle materials and the drugs delivered thereby can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED$_{50}$ (the dose is therapeutically effective in 50% of the population) and LD$_{50}$ (the dose is lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD$_{50}$/ED$_{50}$. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration is performed according to agreed- accepted practices such as standard dosing trials. For example, the therapeutically effective dose can be estimated initially from serum antibody level testing.

The immunogenic compositions of this disclosure can offer various advantages over conventional vaccines, including enhanced immunogenicity of weakly immunogenic antigens, potential reduction in the amount of antigen used, less frequent booster immunizations, improved efficacy, preferential stimulation of immunity, or potential targeting of immune responses. The immunogenic compositions can be administered to a subject by a variety of routes, but preferably by oral administration.

The immunogenic compositions are preferably sterile and contain either a therapeutically or prophylactically effective amount of the immunogenic
compositions in a unit of weight or volume suitable for administration to a subject. The characteristics of the carrier depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

In practicing immunization protocols for treatment and/or prevention of specified diseases, a therapeutically effective amount of the immunogenic composition is administered to a subject. The "effective amount" means the total amount of therapeutic agent (e.g., conjugate) or other active component that is sufficient to show a meaningful benefit to the subject, such as, enhanced immune response, treatment, healing, prevention or amelioration of the relevant medical condition (disease, infection, or the like), or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When "effective amount" is applied to an individual therapeutic agent administered alone, the term refers to that therapeutic agent alone. When applied to a combination, the term refers to combined amounts of the ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. As used herein, the phrase "administering an effective amount" of a therapeutic agent means that the subject is treated with said therapeutic agent(s) in an amount and for a time sufficient to induce an improvement, and preferably a sustained improvement, in at least one indicator that reflects the severity of the disease, infection, or disorder.

An improvement is considered "sustained" if the patient exhibits the improvement on at least two occasions separated by a period of time. The degree of improvement can be determined based, for example, on immunological data, or on signs or symptoms of a disease, infection, or disorder. Various indicators that reflect the extent of the patient's illness can be assessed for determining whether the amount and time of the treatment is sufficient. The baseline value for the chosen indicator or indicators can established based on by examination of the patient prior to administration of the first dose of the therapeutic agent, or based on statistical values generated from a population of healthy patients. If the therapeutic agent is administered to treat acute symptoms, the first dose is administered as soon as practically possible. Improvement is induced by administering therapeutic agents
until the subject manifests an improvement over baseline for the chosen indicator or indicators. In treating chronic conditions, this degree of improvement is obtained by repeatedly administering the therapeutic agents over a period time, e.g., for one, two, or three months or longer, or indefinitely. A single dose can be sufficient for treating or preventing certain conditions. Treatment can be continued indefinitely at the same level or at a reduced dose or frequency, regardless of the patient's condition, if desired. Once treatment has been reduced or discontinued, it later can be resumed at the original level if symptoms reappear.

Generally, the amount of the immunogenic compositions that provides an efficacious dose or therapeutically effective dose for vaccination against infection from bacterial, viral, fungal or parasitic infection is from about 1 µg or less to about 100 mg or more, per kg body weight, such as about 1 µg, 2 µg, 5 µg, 10 µg 15 µg, 25 µg, 50 µg, 100 µg, 250 µg, 500 µg, 1 mg, 2 mg, 5 mg, 10 mg, 15 mg, 25 mg, 50 mg, or 100 mg per kg body weight.

The immunogenic compositions can be administered as a single dose or in a series including one or more boosters. For example, an infant or child can receive a single dose early in life, then be administered a booster dose up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more years later. The booster dose generates antibodies from primed B-cells, for example, an anamnestic response. That is, the immunogenic compositions elicit a high primary functional antibody response in infants or children, and is capable of eliciting an anamnestic response following a booster administration, demonstrating that the protective immune response elicited by the immunogenic compositions is long-lived.

The immunogenic compositions can be formulated into liquid preparations for oral administration. Suitable forms for such administration include suspensions, syrups, and elixirs. Suitable forms for such administration include sterile suspensions and emulsions. Such conjugate vaccines can be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, and the like. The conjugate vaccines can also be lyophilized. The disclosed compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffeting agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of
administration and the preparation desired. Standard texts, such as "Remington: The Science and Practice of Pharmacy", Lippincott Williams & Wilkins; 20th edition (June 1, 2003) and "Remington's Pharmaceutical Sciences", Mack Pub. Co.; 18th and 19th editions (December 1985, and June 1990, respectively), incorporated herein by reference in their entirety, can be consulted to prepare suitable preparations, without undue experimentation. Such preparations can include complexing agents, metal ions, polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, and the like, liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts.

Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lyssolecithin, phospholipids, saponin, bile acids, and the like. The presence of such additional components can influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance, and are thus chosen according to the intended application, such that the characteristics of the carrier are tailored to the selected route of administration.

The compositions can be administered in combination with various vaccines either currently being used or in development, whether intended for human or non-human subjects. Examples of vaccines for human subjects and directed to infectious diseases include the combined diphtheria and tetanus toxoids vaccine; pertussis whole cell vaccine; the inactivated influenza vaccine; the 23-valent pneumococcal vaccine; the live measles vaccine; the live mumps vaccine; live rubella vaccine; Bacille Calmette-Guerin I (BCG) tuberculosis vaccine; hepatitis A vaccine; hepatitis B vaccine; hepatitis C vaccine; rabies vaccine (e.g., human diploid cell vaccine); inactivated polio vaccine; meningococcal polysaccharide vaccine; quadrivalent meningococcal conjugate vaccine; yellow fever live virus vaccine; typhoid killed whole cell vaccine; cholera vaccine; Japanese B encephalitis killed virus vaccine; adenovirus vaccine; cytomegalovirus vaccine; rotavirus vaccine; varicella vaccine; anthrax vaccine; small pox vaccine; and other commercially available and experimental vaccines.
**D. Kits**

The immunogenic compositions disclosed herein can be provided to an administering physician or other health care professional in the form of a kit. The kit is a package which houses a container which contains the immunogenic composition including the vaccine and instructions for administering the composition to a subject. The kit can optionally also contain one or more other therapeutic agents and instructions for use. For example, a vaccine cocktail containing two or more vaccines can be included, or separate pharmaceutical compositions containing different vaccines or therapeutic agents. The kit can also contain separate doses of the composition for serial or sequential administration. The kit can optionally contain instructions for storage, reconstitution (if applicable), and administration of any or all therapeutic agents included. The kits can include a plurality of containers reflecting the number of administrations to be given to a subject. If the kit contains a first and second container, then a plurality of these can be present.

The subject matter of the present disclosure is further illustrated by the following non-limiting Examples.

**EXAMPLES**

**Example 1**

**Materials and Methods**

This example provides the materials and methods used to perform the studies discussed in Examples 2-6 below.

*Animals and reagents.* Female BALB/c (6-8 weeks) were purchased from the Frederick Cancer Research Center (Frederick, MD) or Taconic (Hudson, NY) and housed in pathogen-free conditions in the National Cancer Institute Animal Facility. PCLUS3-18IIIB (SEQ ID NO: 1 KQIINMWQEVGKAMYAPPISGQIRRIQRGPGRAFVTIGK), P18-I10 (SEQ ID NO: 3 RGPGRAFVTI) and OVA_{257-264} (SEQ ID NO: 4 SIINFEKL) were synthesized by NeoMPS (San Diego, CA). TLR ligands including macrophage activating lipoprotein (MALP-2), polyinosine-polycytidylic acid (poly(LC) or PIC) were purchased from Invitrogen (San Diego, CA). Equimolar mixtures of the
phosphorothioate CpG ODNs 1555 and 1466 were used. For *in vitro* stimulation of DCs, MALP2, poly(I:C) and CpG ODN dosed at 0.1 µg/ml, 25 µg/ml and 2 µg/ml, respectively, were added to culture. The vPE16 replicating vaccinia virus was a kind gift of P. Earl and B. Moss. Recombinant vaccinia proteins A33 and L1 were supplied by BEI resources (Manassas, VA) and Gary Cohen.

**PLGA and EUDRAGIT®.** PLGA nanoparticles were manufactured with the NanoDRY® (NANODRY is a registered trademark of NANO-TEX, Inc., Oakland, CA) technology (U.S. Patent Application No. 20050175707). A 0.9 ml of chloroform solution containing 9 mg of PLGA was prepared, followed by addition of 0.1-0.2 mg of FITC-BSA, peptides, or proteins in 0.1 ml of water to form the primary emulsion. The PLGA mixture is emulsified with a Vortex mixer 30 seconds to form the water-in-oil (without) emulsion. The mixture emulsion was added dropwise into 9 ml of isopropanol in a small stainless steel bowl agitated at low frequency sonication (60 hertz, 60% max) with four MOJO beads (1 cm ceramic marbles). Nanoparticles were precipitated at 300 to 400 Torr for 15 minutes at 25°C. The suspension settled for 1 hour in the freezer and the precipitate was lyophilized in a 20-ml glass vial under vacuum overnight. A white powder was typically obtained containing nanoparticles with a diameter of 300 to 400 nm. BSA content/release was determined by BCA or Bradford protein analysis. FITC-BSA release was measured by UV absorbance at 455 nm. Particle size was determined using a Horiba LA-930 Particle Sizer. PLGA and FITC-BSA were purchased from Lakeshore Biomaterials (Birmingham, AL) and Sigma-Aldrich Co. (St. Louis, MO), respectively. FITC-BSA PLGA/FITC-BSA is composed of 1-2% of HTC-BSA and 10 mg of the particle was given i.c.r. to examine mucosal uptake. PLGA encapsulated vaccine is comprised of peptide antigen (PeptAg: 100 µg of PCLUS3-18IIIB per 10 mg of PLGA nanoparticle; ProtAg: A33+L1 10 µg each) and TLR ligands (TLRL: 0.5 µg of MALP-2, 100 µg of poly(LC) and 10 µg of CpG ODN). EUDRAGIT® FS30D or L100-55 (Evonik, Germany) were used to coat PLGA, and 20 mg of microparticles containing 200 µg of PeptAg or 20 µg of A33+L1, 1 µg of MALP-2, 200 µg of poly(LC) and 20 µg of CpG ODN vaccine were used for each oral delivery.
Reagent delivery and virus challenge. For oral delivery, micro/nanoparticles suspended in 50 µl of PBS were given orally through an animal feeding needle. For i.c.r. delivery, reagents were suspended in 100 µl of PBS and delivered with a polished pipette tip through the anal canal. Peptide and TLR ligands as vaccines were mixed with 20 µg of DOTAP liposomal transfection reagent (Roche Diagnostic Corp., Indianapolis, IN) before i.c.r delivery. To challenge mice with vaccinia, 2x10^7 or 1x10^7 PFU of vPE16 were administered by either the i.c.r. or i.vag. route, respectively. vPE16 was recovered from paired ovaries 6 days after challenge. Tissues were homogenized in PBS with a homogenizer (POLYTRON®, Kinematica, Inc., Cincinnati, OH). Plaque-forming assays were performed on CV-I cells for 48 hours followed by counterstaining with 5% w/v crystal violet. Virus presence was expressed as total plaque-forming units (log10 PFUs)/ovaries. To challenge with WR, 4x10^7 or 1x10^7 PFU of the virus were given by either the i.c.r. or i.vag. route, respectively. After WR challenge, mice were weighed for two weeks and euthanized when their weight loss was over 25%.

Electron and fluorescence microscopy. To release nanoparticles from the EUDRAGIT® coating, FS30D/PLGA/Pept+TLR ligands dry particles were incubated with 1-2 ml PBS (pH 7.4) at 37°C for 1.5 hours, and then centrifuged at 2,000 rpm for 2 minutes. The supernatant was concentrated by centrifugation at 10,000 rpm for 10 minutes. The pellet was suspended in a small amount of PBS and placed onto the glow discharged copper grids. After staining with 1% uranyl acetate, the nanoparticles were examined under a FEI CM 120 TEM (equipped with a Gatan GIF100 image filer) operating at a beam energy of 120KeV. Images were acquired by using a Gatan Ik x Ik cooled CCD camera. Field Emission SEM image was acquired. DCs previously incubated with PLGA/FITC-BSA nanoparticles were placed on a glass cover slip and examined for fluorescence expression with a Nikon Eclipse TE-300 inverted fluorescence microscope.

Sample collection. To isolate cells from the gut, different intestines were removed and fragmented into a few pieces and stirred at 37°C with Ca^{2+}- and Mg^{2+}-free HBSS containing 10% FCS, 15 mM HEPES, 5mM EDTA, 0.014% w/v dithiothreitol, and 100 µg/ml gentamycin. Cells from the epithelial layer were
filtered and the remaining fragments were further incubated with complete RPMI 1640 medium containing 250 U/ml collagenase VIII (Sigma Chemical Co., Somerville, NJ) to liberate lamina propria cells.

Bone marrow cells were cultured at 7x10^5 cells/ml for 6 days in the presence of 15 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Sera were collected two weeks after the last immunization. Vaginal washes were collected by pipetting 20 µl of PBS. To prepare homogenates, colon/rectum samples were removed and homogenized in PBS with a homogenizer (POLYTRON, Kinematica, Cincinnati, OH). For resting B-cell purification, spleens were removed from naïve mice and separated by negative separation (to avoid perturbation) on an autoMACS Separator (Miltenyi Biotec Inc., Auburn, CA) using a cocktail of antibodies against CD43, CD4, and Ter-119. The purity of sorted cell populations was at least 97%.

Flow cytometry and antibody ELISA. Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA) or BD Biosciences (San Jose, CA). Soluble tetramer P18-I10 (H-2D^d) was kindly provided by NIH Tetramer Core Facility (Atlanta, GA) and used to determine the frequency of antigen-specific CD8^+ T cells isolated from the intestine. Sample data were acquired on a FACSCalibur and analyzed with FlowJo software (TreeStar Inc, Ashland, OR). To measure TLR ligand activity in stimulating DCs, EUDRAGIT/PLGA/Pept+TLR ligands particles were incubated with PBS (pH 7.4) for 16 hours and centrifuged at 2,000 rpm to remove aggregates. The supernatants were added to DC cultures. After 20 hours of incubation, DCs were stained for intracellular IL-12 with IL-12p70/40 antibodies. For identification of FITC-expressing cells, isolated mucosal cells or in vitro cultured DCs were examined by flow cytometry.

ELISA was performed by serially diluting samples and incubated in plates precoated with WR proteins A33 and L1 (BEI resources, Manassas, VA). The plates were then incubated with HRP-conjugated anti-mouse IgA or IgG detection antibodies (Southern Biotechnology Associates, Birmingham, AL) and color was developed by incubating TMB (Biolegend, San Diego, CA) in darkness. The optical density was read at 450 nm. Antibody titers were derived from the inverse dilution at which the...
sample yielded an optical density twice that of the background of control specimens from unimmunized mice.

Statistical analysis. Comparisons among means of groups were determined by one-way analysis of variance post hoc with Bonferroni correction. Analyses were performed with SPSS (SPSS, Chicago, IL). P-values < 0.05 were considered statistically significant.

Example 2

PLGA nanoparticles enter the mucosa once placed in the large intestine lumen and can induce specific mucosal immune responses.

This example shows that PLGA nanoparticles enter the mucosa once present in the lumen of the large intestine and such particles can induce specific mucosal immune responses.

Uncoated PLGA/FITC-BSA nanoparticles, ranging from 300-500 nm (418 nm ± 88 SD) (FIGS. 3A and 3B), were delivered directly to the colon of mice by i.c.r. administration. At day 2, cells isolated from the large intestinal epithelium were detected positive for fluorescence expression (FIG. 2A), indicating an uptake of the nanoparticles, primarily found in CD1b+B220+ macrophages and secondarily in CD1c+CDIb+ dendritic cells (DCs) (FIG. 7A). Transmission electron microscopy (TEM) indicates PLGA nanoparticles in the cytoplasm (FIG. 7B). Uptake of PLGA nanoparticles by DCs (derived from bone marrow) was also confirmed in vitro by flow cytometry (FIG. 8A), fluorescence microscopy (FIG. 8B) and TEM (FIG. 8C). It was also confirmed that antigen-specific T cells responses could be induced in the colon after a single i.c.r. delivery of PLGA nanoparticles encapsulating PCLUS3-18IIIB (CD4+ T cell helper epitopes fused with HIV Env CD8+ CTL epitope) and TLR ligands (MALP2+poly(I:C)+CpG) (FIG. 2B). These studies demonstrate PLGA nanoparticles can serve as an effective vaccine delivery system once they are deposited in the large intestinal lumen.
Example 3

**EUDRAGIT® microencapsulation enables site-specific uptake of PLGA in the large intestine mucosa**

This example demonstrates site-specific oral delivery of nanoparticle-releasing microparticle vaccines.

The EUDRAGIT® served to make 10-50 μm particles and released contents significantly as early as 1 hour (FIGS. 9A and 9B), while maximal release occurred in 3-4 hours (FIG. 10A). After oral delivery of FS30D containing PLGA/FTTC-BSA nanoparticles (FIG. 10B), nanoparticle uptake was observed almost exclusively in the large intestine (FIGS. 2C and 2D). The cecum is the first part of the large intestine encountered, but in humans, where the cecum is relatively small, the balance between cecum and colon may be different. FS30D was also contrasted with L100-55 microparticles containing the same nanoparticles for lower pH release (pH > 5.5) in the small intestine. Oral administration of EUDRAGIT® L100-55 coated nanoparticles resulted in primary uptake in the small intestine (FIGS. 2C and 2D).

After confirming that formulated vaccine components within the micro/nanoparticles retain TLR agonist activity after release (FIG. 4A), intestinal immune responses were examined in mice after two oral immunizations with the two different EUDRAGIT® coatings of the PLGA nanoparticle vaccine given on day 0 and 14. Three weeks after the second immunization, tetramer positive CD8⁺ T cells were detected in the large intestine, indicating successful induction of colorectal immunity with the oral FS30D-coated vaccine (FIGS. 4B and 4C). In contrast, the L100-55 coated vaccine induced a minimal level of colonic antigen-specific CD8⁺ T cells (HGS. 4B and 4C). In fact, the L100-55-coated vaccine induced a T cell response primarily in the small intestine, where the FS30D-coated vaccine was marginally effective (FIG. 4D). These studies demonstrate that oral administration of FS30D-coated vaccine can successfully induce colorectal immunity.
Example 4

Oral administration of FSSOD/PLGA/vaccine complex induces immune responses and protection against rectal or vaginal viral infection.

This example shows that oral delivery of FS30D-coated PLGA vaccine targeting the large intestine is effective for local vaccination against rectal or vaginal viral infection.

To evaluate the protective efficacy of this vaccine, after prime and boost oral immunization, mice were challenged rectally with a replication-competent vaccinia virus vPE-16, which expresses the HIV Env epitope P18-110 used in the peptide vaccine. Mice immunized with the L100-55-coated vaccine were not protected from the virus challenge. However, the FS30D-coated vaccine reduced viral load almost equally as well as the peptide vaccine given i.c.r. (FIG. 5A). Therefore, oral delivery of FS30D-coated PLGA vaccine targeting the large intestine is effective for local colorectal vaccination against viral infection.

It has been previously shown that i.c.r. vaccination with adenovirus-based vaccines induced effective protection against virus challenge (vaccinia and HSV-2) not only at the rectal but also vaginal mucosa. To determine whether vaginal mucosal protection can be induced by the orally administered FS30D-coated PLGA vaccine, mice were immunized orally with FS30D/PLGA/PCLUS3-18IIIB+TLR ligands twice with a two-week interval, followed by intravaginal (i.vag.) challenge with vPE-16. Compared to i.c.r. immunization, oral delivery of large-intestine targeted FS30D vaccine induced almost equal clearance of virus after vaginal challenge (FIG. 5B), whereas, again, the L100-55 formulation that delivers the vaccine to the small intestine was not effective. Thus, the large intestine was more effective than the small intestine for vaccine delivery against both genital and rectal challenge. This efficacy is largely T-cell mediated, as the virus does not incorporate gpl60 into virions and is not sensitive to anti-gpl60 neutralization.
Example 5

Induction of antibody-mediated immunity against rectal or vaginal viral infection

This example shows induction of antibody-mediated immunity against rectal or vaginal viral infection. The humoral response also plays a role in the gut and genital mucosal immunity. Therefore, it was examined whether encapsulating whole viral proteins in the FS30D coated PLGA vaccine could induce antibody-mediated protective immunity at both mucosal sites after challenging mice with pathogenic vaccinia strain WR. Vaccinia A33 and L1 are immunogens of the extracellular enveloped virion and intracellular mature virion, respectively. Antibody responses induced by the combination of both types of viral antigens encoded by plasmid DNA or as recombinant proteins can protect animals from lethal challenge of WR. Of note, CTLs specific for the vaccinia protein A33 or L1 have not been reported in BALB/c mice. TLR ligands can activate B cells directly and contribute to antigen-specific antibody responses, including in the gut. The triple TLR ligands were evaluated to determine if they synergistically activate B cells (FIG. IA) as determined by CD69. i.c.r. immunization with the combination of recombinant A33 and L1 mixed with the triple TLR ligands in DOTAP induced strong antibody responses in the blood (FIG. 1IB). FS30D coated PLGA containing A33+L1+TLRL vaccine nanoparticles were constructed and administered orally. Vaccinia-specific IgA and IgG antibody responses were significantly induced in both the large intestine (evaluated in tissue homogenates) and vaginal tracts (evaluated in vaginal washes) (FIG. 6A). Further, immunized mice resisted the WR virus challenge by either the rectal or vaginal route (FIG. 6B) as determined by weight loss and by 0 versus 75% or 50% mortality, respectively. Therefore, these studies demonstrate that the FS30D coated PLGA vaccine system can also be used to induce antibody-mediated mucosal protection at both transmission sites. Although the B cell response is still required for the protective immunity even in the presence of CD4+ T cells, the possibility that protein-specific CD4+ T cells could provide help for B cell differentiation and maturation is not to be excluded.
The present examples demonstrate that the disclosed two-part EUDRAGIT®/PLGA nanoparticle-releasing microparticle system can deliver orally administered vaccines highly specifically into the large intestinal mucosa and induce almost equal protective immunity not only in the rectal but also vaginal mucosa. PLGA is a Food and Drug Administration (FDA) approved copolymer for therapeutic purposes owing to its biocompatibility and biodegradability, and long safety record. EUDRAGIT® polymers are also FDA documented copolymers and nontoxic and safe for use. Combinatorial use of these two polymers ensured our orally delivered vaccine’s exclusively targeting the large intestine. These studies indicate that TLR ligand activity was maintained after encapsulation within PLGA. Thus, PLGA is an effective delivery vehicle for mucosal vaccines containing TLR agonists.

Of interest, antigen-specific T cell responses and anti-viral protection in the large intestine were induced only after oral delivery of the PLGA/vaccine coated with the large intestine-targeted FS30D but not the same vaccine coated with the small intestine-targeted L100-55. In contrast, T cell responses were induced in the small intestine by the vaccine with L100-55 but not by the one with FS30D. Thus, these findings strengthen the conception that the common mucosal immune system is somehow sub-compartmentalized. Further, a sub-compartmentalization of the cellular immunity was identified within the GI mucosa immune system. This reciprocal difference in T cell responses in the small and large intestine depending on the site of delivery of the vaccine would have been difficult to detect without a strategy to deliver vaccines selectively to one site or the other, as devised herein. Therefore, the disclosed studies highlight the need for site-specific delivery of vaccines to the large intestinal mucosa for protection against infections transmitted by the rectal or vaginal route, and demonstrate a way to accomplish this via the more practical, non-invasive oral route.

There are many potential applications of the disclosed delivery technology beyond the vaccination against viral infection. This strategy is applicable for many forms of vaccines such as DNA, recombinant proteins, peptides, and a wide variety of adjuvants, and may be adapted in the development of vaccination strategies combating certain sexually transmitted infections caused by not only viruses but
possibly also other types of pathogens. One may also take a new approach to the
development of a preventive or therapeutic vaccination against mucosal
malignancies such as colorectal as well as cervical cancer. With this technology, in-
depth study of the mucosal immunological sub-compartmentalization between the
large and small intestines can be easily conducted. For example, besides
vaccination, one could use this approach to localize the site of induction of oral
tolerance. The technology may be incorporated with other technologies to devise
therapies in which selective targeting within the gut mucosa is needed.

The disclosed targeted micro/nanoparticle system lends itself to practical
large-scale clinical applications because of its 1) great stability in a dry-powder
formulation; 2) easy shipment and storage without refrigeration, and long shelf life;
and 3) economical large batch GMP processing. These features would be highly
desired for effective industrial manufacturing and clinical management.

In conclusion, functional immune compartmentalization of the gut mucosa
has been demonstrated and a nanoparticle-releasing microparticle oral delivery
system was developed and demonstrated to be an easy, non-invasive vaccination
strategy effective against viral infection occurring through the rectal or vaginal
mucosa. Such a vaccination strategy can be used to develop vaccines against
mucosal infections and potentially mucosal cancers and to advanced study of
immunobiological mechanisms involving mucosal compartmentalization. For
example, it is contemplated that this system can be used to design vaccines for HIV,
HPV, HSV, and infections by other infectious species as well as colon cancer,
cervical cancer or other types of mucosal cancers.

Example 6

Immunogens

This example describes specific antigens that can be used as immunogens in
the compositions and methods disclosed herein.

Viral antigens suitable for use with the disclosed methods and compositions
include inactivated (or killed) virus, attenuated virus, split virus formulations,
purified subunit formulations, viral proteins which may be isolated, purified or
derived from a virus, and Virus Like Particles (VLPs). Viral antigens preferably
include epitopes which are exposed on the surface of the virus during at least one stage of its life cycle. Viral antigens include but are not limited to antigens derived from one or more of the viruses set forth below as well as the specific antigen examples identified below.

Viral antigens may be from a Hepatitis C virus (HCV). HCV antigens may be selected from one or more of El, E2, E1/E2, NS345 polyprotein, NS 345-core polyprotein, core, and/or peptides from the nonstructural regions (Houghton et al. (1991) Hepatology 14:381-388, which is incorporated by reference).

Viral antigens may be derived from a Human Herpes virus, such as Herpes Simplex Virus (HSV), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), or Cytomegalovirus (CMV). Human Herpes virus antigens may be selected from immediate early proteins, early proteins, and late proteins. HSV antigens may be derived from HSV-I or HSV-2 strains. HSV antigens may be selected from glycoproteins gB, gC, gD and gH, or immune escape proteins (gC, gE, or gI). VZV antigens may be selected from core, nucleocapsid, tegument, or envelope proteins. A live attenuated VZV vaccine is commercially available. EBV antigens may be selected from early antigen (EA) proteins, viral capsid antigen (VCA), and glycoproteins of the membrane antigen (MA). CMV antigens may be selected from capsid proteins, envelope glycoproteins (such as gB and gH), and tegument proteins.

Exemplary herpes antigens include (GENBANK™ Accession No. in parentheses) those derived from human herpesvirus 1 (Herpes simplex virus type 1) (NC_001806), human herpesvirus 2 (Herpes simplex virus type 2) (NC_001798), human herpesvirus 3 (Varicella zoster virus) (NC_001348), human herpesvirus 4 type 1 (Epstein-Barr virus type 1) (NC_007605), human herpesvirus 4 type 2 (Epstein-Barr virus type 2) (NC_009334), human herpesvirus 5 strain AD169 (NC_001347), human herpesvirus 5 strain Merlin Strain (NC_006273), human herpesvirus 6A (NC_001664), human herpesvirus 6B (NC_000898), human herpesvirus 7 (NC_001716), human herpesvirus 8 type M (NC_003409), and human herpesvirus 8 type P (NC_009333).

Human Papilloma virus (HPV) antigens are known in the art and can be found for example in International Patent Publication No. WO96/19496, (incorporated by reference in its entirety) which discloses variants of HPV E6 and
E7 proteins, particularly fusion proteins of E6/E7 with a deletion in both the E6 and E7 proteins. HPV L1 based antigens are disclosed in international Patent publication Nos. W094/00152, W094/20137, W093/02184 and W094/05792, all of which are incorporated by reference. Such an antigen can include the L1 antigen as a monomer, a capsomer or a virus like particle. Such particles may additionally comprise L2 proteins. Other HPV antigens are the early proteins, such as E7 or fusion proteins such as L2-E7. Exemplary HPV antigens include (GENBANK™ Accession No. in parentheses) those derived from human papillomavirus-1 (NC_001356), human papillomavirus-18 (NC_001357), human papillomavirus-2 (NC_001352), human papillomavirus-54 (NC_001676), human papillomavirus-61 (NC_001694), human papillomavirus-cand90 (NC_004104), human papillomavirus RTRX7 (NC_004761), human papillomavirus type 10 (NC_001576), human papillomavirus type 101 (NC_008189), human papillomavirus type 103 (NC_008188), human papillomavirus type 107 (NC_009239), human papillomavirus type 16 (NC_001526), human papillomavirus type 24 (NC_001683), human papillomavirus type 26 (NC_001583), human papillomavirus type 32 (NC_001586), human papillomavirus type 34 (NC_001587), human papillomavirus type 4 (NC_001457), human papillomavirus type 41 (NC_001354), human papillomavirus type 48 (NC_001690), human papillomavirus type 49 (NC_001591), human papillomavirus type 5 (NC_001531), human papillomavirus type 50 (NC_001691), human papillomavirus type 53 (NC_001593), human papillomavirus type 60 (NC_001693), human papillomavirus type 63 (NC_001458), human papillomavirus type 6b (NC_001355), human papillomavirus type 7 (NC_001595), human papillomavirus type 71 (NC_002644), human papillomavirus type 9 (NC_001596), human papillomavirus type 92 (NC_004500), and human papillomavirus type 96 (NC_005134).

Viral antigens may be derived from a Retrovirus, such as an Oncovirus, a Lentivirus or a Spumavirus. Oncovirus antigens may be derived from HTLV-I, HTLV-2 or HTLV-5. Lentivirus antigens may be derived from HIV-I or HIV-2. Retrovirus antigens may be selected from gag, pol, env, tax, tat, rex, rev, nef, vif, vpu, and vpr. Antigens for HIV are known in the art, for example HIV antigens may be selected from gag (p24gag and p55gag), env (gpl60 and gp41), pol, tat, nef, rev
vpu, miniproteins, (p55 gag and gpl40v). HIV antigens may be derived from one or more of the following strains: HIVmb, HIV; HIVLAV, HIVLAI, HIVM N, HIV-I CM235, HIV-I US4. Examples of HIV antigens can be found in International Patent Publication Nos. WO09/089568, WO09/080719, WO08/099284, and WO00/15255, and U.S. Patent No. 7,531,181 and 6,225,443, all of which are incorporated by reference. Exemplary HIV antigens include (GENBANK™ Accession No. in parentheses) those derived from human immunodeficiency virus 1 (NC_001802), human immunodeficiency virus 2 (NC_001722).

Bacterial antigens suitable for use in the disclosed methods and compositions include proteins, polysaccharides, lipopolysaccharides, and outer membrane vesicles which may be isolated, purified or derived from a bacterium. In addition, bacterial antigens include bacterial lysates and inactivated bacteria formulations. Bacteria antigens can be produced by recombinant expression. Bacterial antigens preferably include epitopes which are exposed on the surface of the bacteria during at least one stage of its life cycle. Bacterial antigens include but are not limited to antigens derived from one or more of the bacteria set forth below as well as the specific antigens examples identified below.

*Neisseria gonorrhoeae* antigens include Por (or porin) protein, such as PorB (see, e.g., Zhu et al. (2004) *Vaccine* 22:660-669), a transferring binding protein, such as TbpA and TbpB (see, e.g., Price et al. (2004) *Infect. Immun.* 71(l):277-283), an opacity protein (such as Opa), a reduction-modifiable protein (Rmp), and outer membrane vesicle (OMV) preparations (see, e.g., Plante et al. (2000) *J. Infect. Dis.* 182:848-855); WO 99/24578; WO 99/36544; WO 99/57280; and WO 02/079243, all of which are incorporated by reference).

*Chlamydia trachomatis* antigens include antigens derived from serotypes A, B, Ba and C (agents of trachoma, a cause of blindness), serotypes Li, L3 (associated with Lymphogranuloma venereum), and serotypes, D-K. *Chlamydia trachomas* antigens also include antigens identified in WO 00/37494; WO 03/049762; WO 03/068811; and WO 05/002619 (all of which are incorporated by reference), including PepA (CT045), LcrE (CT089), Art (CT381), DnaK (CT396), CT398, OmpH-like (CT242), L7/L12 (CT316), OmcA (CT444), AtosS (CT467), CT547,
Eno (CT587), HrtA (CT823), MurG (CT761), CT396 and CT761, and specific combinations of these antigens.

*Treponemapallidum* (Syphilis) antigens include TmpA antigen.

The compositions of the invention can include one or more antigens derived from a sexually transmitted disease (STD). Such antigens can provide for prophylactic or therapy for STDs such as chlamydia, genital herpes, hepatitis (such as HCV), genital warts, gonorrhea, syphilis and/or chancroid (see WO 00/15255, which is incorporated by reference). Antigens may be derived from one or more viral or bacterial STDs. Viral STD antigens for use in the invention may be derived from, for example, HIV, herpes simplex virus (HSV-I and HSV-2), human papillomavirus (HPV), and hepatitis (HCV). Bacterial STD antigens for use in the invention may be derived from, for example, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponemapallidum*, *Haemophilus ducreyi*, *E. coli*, and *Streptococcus agalactiae*.


**EXAMPLE 7**

**Treatment of a Mucosal Cancer in a Human**

This example describes a particular method that can be used to treat a mucosal tumor in humans by use of the disclosed oral vaccine system. Although particular methods, dosages, and modes of administrations are provided, one skilled in the art will appreciate that variations can be made without substantially affecting the treatment.

Based upon the teaching disclosed herein, a mucosal cancer, such as colonic or cervical cancer, can be treated by administering a therapeutically effective
amount of a disclosed immunogenic composition comprising one or more tumor-associated antigens, such as mucosal cancer-associated antigens.

Briefly, the method can include screening subjects to determine if they have a tumor, such as a mucosal cancer (e.g., cervical or colonic). Subjects having a mucosal cancer are selected. In one example, a clinical trial would include half of the subjects following the established protocol for treatment of mucosal cancer (such as a normal chemotherapy/radiotherapy/surgery regimen). The other half would follow the established protocol for treatment of the tumor (such as a normal chemotherapy/radiotherapy/surgery regimen) in combination with administration of the immunogenic compositions described above. In some examples, the tumor is surgically excised (in whole or part) prior to treatment with the immunogenic compositions. In another example, a clinical trial would include half of the subjects following the established protocol for treatment of mucosal cancer (such as a normal chemotherapy/radiotherapy/surgery regimen). The other half would follow the administration of the immunogenic compositions described above. In some examples, the tumor is surgically excised (in whole or part) prior to treatment with the therapeutic compositions.

**Screening subjects**

In some examples, the subject is first screened to determine if they have a mucosal cancer or are at risk of acquiring one. In particular examples, the subject is screened to determine if they have colonic or cervical cancer. Examples of methods that can be used to screening for a mucosal cancer include a combination of ultrasound, tissue biopsy, and serum blood levels. If blood or a fraction thereof (such as serum) is used, 1-100 µl of blood is collected. Serum can either be used directly or fractionated using filter cut-offs to remove high molecular weight proteins. If desired, the serum can be frozen and thawed before use. If a tissue biopsy sample is used, 1-100 µg of tissue is obtained, for example using a fine needle aspirate.

In some examples, the biological sample (e.g., tissue biopsy or serum) is analyzed to determine if it expresses one or more molecules known to be associated with a particular type of mucosal cancer, such as colon or cervical cancer. Expression of one or more molecules associated with mucosal cancer is indicative
that the subject is a candidate for receiving the therapeutic compositions disclosed herein. However, such pre-screening is not required prior to administration of the therapeutic compositions disclosed herein.

Pre-treatment of subjects

In particular examples, the subject is treated prior to administration of a immunogenic composition for the treatment of a mucosal cancer. However, such pre-treatment is not always required, and can be determined by a skilled clinician. For example, the tumor can be surgically excised (in total or in part) prior to administration of one or more immunogenic compositions. In addition, the subject can be treated with an established protocol for treatment of the particular tumor present (such as a normal chemotherapy/radiotherapy regimen).

Administration of therapeutic compositions

Following subject selection, a therapeutic effective dose of the immunogenic composition is administered to the subject. For example, a therapeutic effective dose of an agent to one or more of the disclosed immunogenic compositions is orally administered to the subject to reduce or inhibit tumor growth and/or vascularization.

The amount of the composition administered to prevent, reduce, inhibit, and/or treat a mucosal cancer or a condition associated with it depends on the subject being treated, the severity of the disorder, and the manner of administration of the therapeutic composition. Ideally, a therapeutically effective amount of an agent is the amount sufficient to prevent, reduce, and/or inhibit, and/or treat the condition (e.g., mucosal cancer) in a subject without causing a substantial cytotoxic effect in the subject. An effective amount can be readily determined by one skilled in the art, for example using routine trials establishing dose response curves. In addition, particular exemplary dosages are provided above. The immunogenic compositions can be administered in a single dose delivery or in a repeated administration protocol (for example, by a, daily, weekly, or monthly repeated administration protocol). As such, these compositions may be formulated with an inert diluent or with a pharmaceutically acceptable carrier.

In one specific example, the bioactive agent is a tumor-associated antigen. Tumor antigens are proteins that are produced by tumor cells that elicit an immune response, particularly T-cell mediated immune responses. The tumor antigen can be
any tumor-associated antigen, which are well known in the art and include, for example, carciñoembryonic antigen (CEA), β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-I, MN-CA IX, human telomerase reverse transcriptase, R乌, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, macrophage colony stimulating factor, prostate, prostate-specific antigen (PSA), PAP, NY-ESO-I, LAGE-Ia, p53, prostein, PSMA, Her2/neu, survivin and telomerase, prostate-carcinoma tumor antigen-1, MAGE, ELF2M, neutrophil elastase, ephrinB2, CD22, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor and mesothelin. A list of selected tumor antigens and their associated tumors are shown below in Table 2.

**Table 2**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor Associated Target Antigens</th>
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<tbody>
<tr>
<td>Acute myelogenous leukemia</td>
<td>Wilms tumor 1 (WT1), PRAME, PR1, proteinase 3, elastase, cathepsin G</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia</td>
<td>WT1, PRAME, PR1, proteinase 3, elastase, cathepsin G</td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>WT1, PRAME, PR1, proteinase 3, elastase, cathepsin G</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>PRAME</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>Survivin</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>Survivin</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>NY-ESO-1</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>MAGE, MART, Tyrosinase, PRAME GP100</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>WT1, Herceptin, epithelial tumor antigen (ETA)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>WT1</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>CA-125</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>PSA</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>CA19-9, RCAS1</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>CEA</td>
</tr>
<tr>
<td>Cervical Cancer</td>
<td>SCC, CA125, CEA, Cytokeratins (TPA, TPS, Cyfra21-1)</td>
</tr>
<tr>
<td>Renal cell carcinoma (RCC)</td>
<td>Fibroblast growth factor 5</td>
</tr>
<tr>
<td>Germ cell tumors</td>
<td>AFP</td>
</tr>
</tbody>
</table>

In some embodiments, a subject is administered an immunogenic composition including one or more tumor-associated antigens following diagnosis of the subject (e.g. a diagnosis of the cancer). The immunogenic composition can be administered in a single dose or in multiple doses over time. In some examples, a subject having a mucosal cancer is administered an immunogenic composition orally daily for at least one week, at least one month or at least three months.
Administration of the immunogenic compositions can be continued after chemotherapy and radiation therapy is stopped and can be taken long term (for example over a period of months or years).

Assessment

Following the administration of one or more therapies, subjects having a tumor (for example, a mucosal cancer) can be monitored for tumor treatment, such as regression or reduction in metastatic lesions, tumor growth or vascularization. In particular examples, subjects are analyzed one or more times, starting 7 days following treatment. Subjects can be monitored using any method known in the art.

For example, diagnostic imaging can be used (such as x-rays, CT scans, MRIs, ultrasound, fiber optic examination, and laparoscopic examination), as well as analysis of biological samples from the subject (for example analysis of blood, tissue biopsy, or other biological samples), such as analysis of the type of cells present, or analysis for a particular tumor marker. In one example, if the subject has advanced mucosal cancer, assessment can be made using ultrasound, MRI, or CAT scans, or analysis of the type of cells contained in a tissue biopsy. It is also contemplated that subjects can be monitored for the response of their tumor(s) to therapy during therapeutic treatment by at least the aforementioned methods.

Additional treatments

In particular examples, if subjects are stable or have a minor, mixed or partial response to treatment, they can be re-treated after re-evaluation with the same schedule and preparation of agents that they previously received for the desired amount of time, such as up to a year of total therapy. A partial response is a reduction in size or growth of some tumors, but an increase in others.

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that illustrated embodiments are only examples of the invention and should not be considered a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.
We claim:

1. An immunogenic composition formulated for oral administration, comprising:
   nanoparticles comprising a polymer core encapsulating a therapeutically effective amount of a target immunogen and a therapeutically effective amount of one or more immunostimulatory agents; and
   a microparticle carrier encapsulating multiple nanoparticles within the microparticle carrier, wherein the carrier comprises a pH-sensitive copolymer matrix that is insoluble in acidic media, but dissolves by salt formation above pH 6.5, thereby forming an immunogenic composition capable of eliciting an immune response in a subject in the intestine.

2. The immunogenic composition of claim 1, wherein the target immunogen is one or more antigens present in a pathogen of interest.

3. The immunogenic composition of claim 2, wherein the pathogen of interest is a bacterial, viral, fungal or parasitic pathogen of interest.

4. The immunogenic composition of claim 2 or 3, wherein the pathogen of interest causes a sexually transmitted disease.

5. The immunogenic composition of any one of claims 1-4, wherein the target immunogen is an antigenic polypeptide.

6. The immunogenic composition of any one of claims 1-5, wherein the target immunogen comprises one or more HIV envelope CTL polypeptide.

7. The immunogenic composition of claim 6, wherein the one or more HIV envelope CTL polypeptide comprises a gp41 or gp120 polypeptide.

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8. The immunogenic composition of claim 7, wherein the one or more HIV envelope CTL polypeptide is a PCLUS3-18IIIB peptide.

9. The immunogenic composition of any one of claims 1-8, wherein the polymer core comprises at least one polyhydroxy acid.

10. The immunogenic composition of claim 9, wherein the at least one polyhydroxy acid is selected from the group consisting of polylactic acid (PLA), polyglycolic acid (PGA), their copolymers poly(lactic-co-glycolic acid) (PLGA).

11. The immunogenic composition of any one of claims 1-10, wherein the one or more immunostimulatory agents comprises one or more Toll-like receptor (TLR) ligands.

12. The immunogenic composition of claim 11, wherein the one or more TLR ligands is MALP2, poly(I:C), CpG 2343 or combination thereof.

13. The immunogenic composition of claim 11, wherein the one or more TLR ligands comprises three Toll-like receptor (TLR) ligands.

14. The immunogenic composition of claim 13, wherein the three TLR ligands are MALP2, poly(LC), and CpG 2343.

15. The immunogenic composition of any one of claims 1-14, wherein the one or more immunostimulatory agents comprises one or more cytokines such as but not limited to IL-15, IL-12 or GM-CSF.

16. The immunogenic composition of any one of claims 1-15, wherein the pH-sensitive copolymer matrix is an anionic copolymer.

17. The immunogenic composition of claim 16, wherein the anionic copolymer comprises methyl acrylate, methyl methacrylate and methacrylic acid.
18. The immunogenic composition of any one of claims 1-17, wherein the polymer core comprises nanoparticles having a size between about 10 and about 1000 nm.

19. The immunogenic composition of claim 18, wherein the polymer core comprises nanoparticles having a size between 100 and about 500 nm.

20. The immunogenic composition of any one of claims 1-19, wherein the pH-sensitive copolymer matrix results in particles having a size between about 1 and about 100 micrometers.

21. The immunogenic composition of claim 20, wherein the particle size is between about 1 and about 10 micrometers.

22. The immunogenic composition of any one of claims 1-21, wherein the target immunogen prevents, inhibits or reduces a sexually transmitted disease or infection.

23. A method of treating and/or inhibiting an infection by a pathogen of interest in a subject, comprising:

   selecting a subject for treatment that has, or is at risk for developing, an infection by a pathogen of interest; and

   orally administering to the subject a therapeutically effective amount of the immunogenic composition of any one of claims 1-22, wherein the bioactive agent in the immunogenic composition comprises one or more antigens present in the pathogen of interest, thereby treating and/or inhibiting an infection by the pathogen of interest in the subject.

24. The method of claim 23, wherein the pathogen of interest causes a sexually transmitted disease.
25. A method of inducing a mucosal immune response in the large intestine of a subject, comprising:
   orally administering to the subject a therapeutically effective amount of the immunogenic compositions of any one of claims 1-22, thereby generating the mucosal immune response in the large intestine of the subject.

26. A method for treating or preventing a sexually transmitted disease of interest in a subject, comprising:
   orally administering to the subject a therapeutically effective amount of the immunogenic composition of any one of claims 1-22, thereby treating the subject or preventing infection of the subject with the sexually transmitted disease.

27. A method of treating a mucosal cancer in a subject, comprising:
   selecting a subject with a mucosal cancer; and
   orally administering to the subject a therapeutically effective amount of an immunogenic composition comprising nanoparticles comprising a polymer core encapsulating a therapeutically effective amount of a tumor antigen and a therapeutically effective amount of one or more immunostimulatory agents; and a microparticle carrier encapsulating multiple nanoparticles within the microparticle carrier, wherein the carrier comprises a pH-sensitive copolymer matrix that is insoluble in acidic media, but dissolves by salt formation above pH 6.5,
   thereby inducing an immune response to the mucosal cancer in the subject and treating the mucosal cancer.
### FIG. 7A

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<th>Untreated</th>
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<tr>
<td></td>
<td>All</td>
<td>FITC+</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>2.96</td>
<td>0.32</td>
<td>4.05</td>
</tr>
<tr>
<td>CD11b</td>
<td>0.33</td>
<td>0.003</td>
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<td>0.0006</td>
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<td>CD11c</td>
<td>3.45</td>
<td>0.003</td>
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<tr>
<td></td>
<td>0.7</td>
<td>0.0003</td>
<td>0.38</td>
</tr>
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</table>

### FIG. 7B

![Image of cell structures](image-url)
FIG. 11A

CD69^+ cells (%)

MALP2
Poly(I:C)
CpG

FIG. 11B

IgG titer

A33+L1
TLRL
DOTAP

12/12
A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/16 A61K9/51 A61K38/00 A61K39/00 C07K14/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Further documents are listed in the continuation of Box C X See patent family annex

Special categories of cited documents
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"S" document member of the same patent family

Date of the actual completion of the international search
12 November 2010

Date of mailing of the international search report
23/11/2010

Name and mailing address of the ISA/
European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax (+31-70) 340-3016

Gimenez Miral les, J
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