

## CHIMERIC ANTIGENS FOR BREAKING HOST TOLERANCE TO FOREIGN ANTIGENS

### 5 I. INTRODUCTION

#### A. Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/493,449, filed August 8, 2003, which is herein incorporated by reference.

#### B. Technical Field

10 The present invention relates to methods and compositions for eliciting or enhancing an immune response and for breaking host tolerance to foreign antigens.

#### C. Background

When a healthy host (human or animal) encounters a foreign antigen, such as a protein derived from a bacterium, virus and/or parasite, the host normally initiates an  
15 immune response. This immune response can be a humoral response and/or a cellular response. In the humoral response, antibodies are produced by B cells and are secreted into the blood and/or lymph in response to an antigenic stimulus. The antibody then neutralizes the antigen, *e.g.* a virus, by binding specifically to antigens on its surface, marking it for destruction by phagocytotic cells and/or complement-  
20 mediated mechanisms, or by blocking binding or by enhancing clearance of free antigen from circulation. The cellular response is characterized by the selection and expansion of specific helper and cytotoxic T-lymphocytes capable of directly or indirectly eliminating the cells that contain the antigen.

In some individuals, the immune system fails to respond to certain foreign  
25 antigens. When an antigen does not stimulate the production of specific antibodies and/or killer T cells, the immune system is unable to prevent the resultant disease. Thus, the infectious agent, *e.g.* a virus, can establish a chronic infection and the host immune system becomes tolerant to the antigens produced by the infectious agent.

While the mechanism by which the infectious agent evades the host immune  
30 machinery is not clearly established, the lack of proper presentation of foreign antigens to the host immune system may be a contributing factor to development of

chronic infections. Antigen presenting cells (APCs) process the encountered antigens differently depending on the localization of the antigen. Exogenous antigens are endocytosed and subsequently processed within the endosomes of the antigen presenting cell. The peptide fragments generated from exogenous antigens are presented on the surface of the cell complexed with Major Histocompatibility Complex (MHC) Class II. The presentation of this complex to CD4<sup>+</sup> T cells stimulates the CD4<sup>+</sup> T helper cells to secrete cytokines that stimulate B cells to produce antibodies against the exogenous antigen (humoral response). Intracellular antigens, on the other hand, are processed and presented as complexes with MHC Class I on the surface of antigen presenting cells. Antigen presentation to CD8<sup>+</sup> T cells results in a cytotoxic T cell (CTL) immune response against host cells that carry the antigen.

In subjects with chronic viral or parasitic infections (where the organism is resident inside a host cell at some point during its life cycle), antigens will be produced by and expressed in the host cell and secreted antigens will be present in the circulation. As an example, in the case of a chronic human hepatitis B virus (HBV) carrier, virions and the HBV surface antigens and a surrogate of core antigens (in the form of the e-antigen) can be detected in the blood.

An effective therapy for a chronic infection requires a strong CTL response against antigens associated with the infectious agent. This can be achieved either by producing the antigen within the host cell, or delivering the antigen to the appropriate cellular compartment so that it gets processed and presented so as to elicit a cellular response. Several approaches have been documented in the literature to deliver an antigen intracellularly. Among these, viral vectors (Lorenz et al., *Hum. Gen. Ther.* 10:623-631 (1999)), the use of cDNA-transfected cells (Donnelly et al., *Ann. Rev. Immunol.* 15:617(1997)) as well as the expression of the antigen through injected cDNA vectors (Lai et al., *Crit. Rev. Immunol.* 18:449-484 (1988); and US Patent No. 5,589,466), have been documented. Further, DNA vaccines expressing antigens targeted to dendritic cells have been described (You, et al., *Cancer Res* 61:3704-3711 (2001)).

Delivery vehicles capable of carrying the antigens to the cytosolic compartment of the cell for MHC Class I pathway processing have also been used. Hilgers, et al. (*Vaccine* 17: 219-228 (1999)) have described in detail the use of

adjuvants to achieve the same goal. Another approach is the use of biodegradable microspheres for cytoplasmic delivery of antigens, exemplified by the generation of a Th1 immune response against ovalbumin peptide (Newman, et al., *J Control Release* 54:49-59 (1998); and Newman, et al., *J Biomed Mater Res* 50:591-597 (2000)).

5 Additionally, antigen presenting cells, e.g., dendritic cells, take up PLGA nanospheres (Newman, et al., *J Biomed Mater Res* 60:480-486 (2002)).

The ability of dendritic cells to capture, process, and present the antigen and to stimulate naïve T cells has made them very important tools for therapeutic vaccine development (Laupeze, et al., *Hum Immunol* 60:591-597 (1999)). Targeting of the

10 antigen to the dendritic cells is a crucial step in antigen presentation and the presence of several receptors on dendritic cells for the Fc region of antibodies have been exploited for this purpose (Regnault, et al., *J Exp Med* 189:371-380 (1999)). Additional examples of this approach include ovarian cancer Mab-B43.13, Anti-PSA antibody as well as Anti-HBV antibody antigen complexes (Wen, et al., *Int Rev*

15 *Immunol* 18:251-258 (1999)). Cancer immunotherapy using dendritic cells loaded with tumor associated antigens has been shown to produce tumor-specific immune responses and anti-tumor activity (Fong and Engleman, *Ann Rev Immunol* 96:1865-1972 (2000); and Campton, et al. *J Invest Dermatol* 115:57-61 (2000)). Promising results were obtained in clinical trials *in vivo* using tumor-antigen-pulsed dendritic

20 cells (Tarte and Klein, *Leukemia* 13:653-663 (1999)). These studies clearly demonstrate the efficacy of using dendritic cells to generate immune responses against cancer antigens.

Antigen presentation can also be affected via mannose receptors, in place of, or in addition to, utilizing the Fc receptor on antigen presenting cells. The

25 macrophage mannose receptor (MMR), also known as CD206, is expressed on antigen presenting cells such as dendritic cells (DC). This molecule is a member of the C-type lectin family of endocytic receptors. Mannosylated antigens can be bound and internalized by CD206. In general, exogenous antigen is thought to be processed and presented primarily through the MHC class II pathway. However, in the case of

30 targeting through CD206, there is evidence that both the MHC class I and class II pathways are involved (Apostolopoulos et al., *Eur. J. Immunol.* 30:1714 (2000); Apostolopoulos and McKenzie, *Curr. Mol. Med.* 1:469 (2001); Ramakrishna et al., *J. Immunol.* 172:2845-2852 (2004)).

Infectious disease and cancer are major public healthcare issues. For example, World Health Organization statistics show that more than 2 billion people have been infected by HBV. Among these, 370 million are chronically infected and, as a result, have a high probability of developing cirrhosis of the liver and hepatocellular carcinoma. Approximately 170 million people worldwide are chronic carriers of HCV, for which there is no effective prophylactic or therapeutic vaccine. The World Health Organization reports that 10 million people are diagnosed with cancer every year. Cancer causes 6 million deaths every year, 12% of deaths worldwide. Thus a need exists for new, therapeutically effective compositions and methods for the eliciting immune responses against infections and cancer, as well as new methods for producing such compositions.

## II. SUMMARY OF THE INVENTION

The invention provides chimeric antigens for eliciting an immune response, the chimeric antigens comprising an immune response domain and a target binding domain, wherein the target binding domain comprises an antibody fragment.

Another aspect of the invention provides methods of enhancing antigen presentation in an antigen presenting cell comprising contacting the antigen presenting cell with a composition comprising a chimeric antigen of the invention.

Yet another aspect of the invention provides methods of activating antigen presenting cells comprising contacting the antigen presenting cells with a chimeric antigen of the invention.

An aspect of the invention provides methods of eliciting an immune response comprising administering to a subject, a composition comprising a chimeric antigen of the invention.

Another aspect of the invention provides methods of breaking tolerance comprising administering a chimeric antigen of the invention to a subject. In a preferred embodiment, the subject is chronically infected with a virus or an obligate intracellular parasite.

One aspect of the invention provides methods of treating an immune-treatable condition comprising administering, to a subject in need thereof, a therapeutically effective amount of a chimeric antigen of the invention. In a preferred embodiment,

the immune-treatable condition is an infection, especially a chronic infection, or a cancer.

Yet another aspect of the invention provides methods of vaccinating a subject against an infection comprising administering a chimeric antigen of the present invention to the subject. The subject can be prophylactically or therapeutically vaccinated. In a preferred embodiment, the subject develops an immune response to more than one epitope of the chimeric antigen, and more preferably to more than one epitope of the immune response domain. Preferably, the infection is a viral infection or an obligate intracellular parasitic infection.

Another aspect of the invention provides a pharmaceutical composition comprising a chimeric antigen of the present invention and a pharmaceutically acceptable excipient.

An aspect of the invention provides articles of manufacture comprising a chimeric antigen of the invention and instructions for administering the chimeric antigen to a subject in need thereof.

Another aspect of the invention provides polynucleotides encoding a chimeric antigen, said polynucleotide comprising a first polynucleotide portion encoding an immune response domain and a second polynucleotide portion encoding a target binding domain, wherein the target binding domain comprises an antibody fragment. The invention also provides microorganisms and cell lines comprising such polynucleotides.

Yet another aspect of the invention provides methods of producing a chimeric antigen of the invention comprising providing a microorganism or cell line, which comprises a polynucleotide that encodes a chimeric antigen of the invention, and culturing the microorganism or cell line under conditions whereby the chimeric antigen is expressed.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

### III. DESCRIPTION OF DRAWINGS

FIG. 1A provides a schematic diagram illustrating the structure of a chimeric antigen of the present invention as a monomer, wherein the chimeric antigen has two portions, namely an immune response domain and a target binding domain. The schematic also illustrates a preferred embodiment, in which a hinge region is present. FIG. 1B provides a schematic diagram illustrating the structure of a chimeric antigen of invention in its normal state, assembled as a dimer. This schematic illustrates a particularly preferred embodiment, in which the chimeric antigen comprises a 6xHis tag and peptide linker in addition to the immune response and target binding domains.

FIG. 2 demonstrates that stimulation of T cells with HBV S1/S2-TBD generates a cytotoxic T cell (CTL) response specifically against an epitope from the HBV S1 protein.

FIG. 3 demonstrates that stimulation of T cells with HBV S1/S2-TBD generates a cytotoxic T cell (CTL) response specifically against an epitope from the HBV S2 protein.

FIG. 4 shows the comparison of uptake of HBV S1/S2-TBD, IgG1, and IgG2 by maturing dendritic cells as a function of concentration.

FIG. 5 shows the correlation of HBV S1/S2-TBD binding to CD32 and CD206 expression on dendritic cells.

### IV. DETAILED DESCRIPTION

#### A. Overview

Disclosed herein are compositions and methods for eliciting immune responses against antigens. In particular, the compounds and methods elicit immune responses against foreign antigens that are otherwise recognized by the host as "self" antigens, thus breaking host tolerance to those antigens. Presenting the host immune system with a chimeric antigen comprising an immune response domain and a target binding domain, wherein the target binding domain comprises an antibody fragment, enhances the immune response against the foreign or tolerated antigen. Antigen presenting cells take up, process, and present the chimeric antigen, eliciting both a humoral and cellular immune response against the desired antigen.

## B. Definitions

Prior to describing the invention in further detail, the terms used in this application are defined as follows unless otherwise indicated.

"Antibody" refers to an immunoglobulin molecule produced by B lymphoid  
5 cells with a specific amino acid sequence evoked in humans or other animals by an antigen (immunogen). These molecules are characterized by reacting specifically with the antigen, each being defined in terms of the other.

"Antibody response" or "humoral response" refers to a type of immune response in which antibodies are produced by B lymphoid cells and are secreted into  
10 the blood and/or lymph in response to an antigenic stimulus. In a properly functioning immune response, the antibody binds specifically to antigens on the surface of cells (e.g., a pathogen), marking the cell for destruction by phagocytotic cells and/or complement-mediated mechanisms. Antibodies also circulate systemically and can bind to free virions. This antibody binding can neutralize the virion and prevent it  
15 from infecting a cell as well as marking the virion for elimination from circulation by phagocytosis or filtration in the kidneys.

"Antigen" refers to any substance that, as a result of coming in contact with appropriate cells, induces a state of sensitivity and/or immune responsiveness and that reacts in a demonstrable way with antibodies and/or immune cells of the sensitized  
20 subject *in vivo* or *ex vivo*.

"Antigen-presenting cell" refers to the accessory cells of antigen inductive events that function primarily by handling and presenting antigen to lymphocytes. The interaction of antigen presenting cells with antigens is an essential step in immune induction because it enables lymphocytes to encounter and recognize antigenic  
25 molecules and to become activated. Exemplary antigen presenting cells include macrophages, Langerhans-dendritic cells, Follicular dendritic cells, and B cells.

"B cell" refers to a type of lymphocyte that produces immunoglobulins or antibodies that interact with antigens.

"C<sub>H</sub>1 region" refers to a region of the heavy chain constant domain on the  
30 antigen binding fragment of an antibody.

"Cellular response" refers to a type of immune response mediated by specific helper and killer T cells capable of directly eliminating virally infected or cancerous cells.

As used herein, the term "chimeric antigen" refers to a polypeptide comprising an immune response domain and a target binding domain. The immune response domain and target binding domains may be directly or indirectly linked by covalent or non-covalent means.

5        "Cytotoxic T-lymphocyte" is a specialized type of lymphocyte capable of destroying foreign cells and host cells infected with the infectious agents that produce viral antigens.

      "Epitope" refers to the simplest form of an antigenic determinant, on a complex antigen molecule; this is the specific portion of an antigen that is recognized  
10 by an immunoglobulin or T cell receptor.

      "Fusion protein" refers to a protein formed by expression of a hybrid gene made by combining two or more gene sequences.

      "Hinge region" refers to the portion of an antibody that connects the Fab fragment to the Fc fragment; the hinge region contains disulfide bonds that covalently  
15 link the two heavy chains.

      The term "homolog" refers to a molecule which exhibits homology to another molecule, by for example, having sequences of chemical residues that are the same or similar at corresponding positions. The phrase "% homologous" or "% homology" refers to the percent of nucleotides or amino acids at the same position of homologous polynucleotides or polypeptides that are identical or similar. For example, if 75 of 80  
20 residues in two proteins are identical, the two proteins are 93.75% homologous. Percent homology can be determined using various software programs known to one of skill in the art.

      "Host" refers to a warm-blooded animal, including a human, which suffers  
25 from an immune-treatable condition, such as an infection or a cancer. As used herein, "host" also refers to a warm-blooded animal, including a human, to which a chimeric antigen is administered.

      In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, the preferred mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary  
30 nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleobases that pair through

the formation of hydrogen bonds. Hybridization can occur under varying circumstances. The terms "hybridize", "hybridizing", "hybridizes" and the like, used in the context of polynucleotides, are meant to refer to conventional hybridization conditions, preferably such as hybridization in 50% formamide/6X SSC/0.1% SDS/100 µg/ml mDNA, in which temperatures for hybridization are above 37°C and temperatures for washing in 0.1X SSC/0.1% SDS are above 55°C.

"Immunity" or "immune response" refers to the body's response to an antigen. In particular embodiments, it refers to the ability of the body to resist or protect itself against infectious disease.

10        "Immune Response Domain (IRD)" refers to the variously configured antigenic portion of the bifunctional chimeric antigen molecule. The immune response domain comprises one or more antigens and/or one or more recombinant antigens.

As used herein, the phrase "immune-treatable condition" refers to a condition or disease that can be prevented, inhibited or relieved by eliciting or modulating an immune response in the subject.

"Lymphocyte" refers to a subset of nucleated cells found in the blood, which mediate specific immune responses.

20        "Monoclonal antibody" or "mAb" refers to an antibody produced from a clone or genetically homogenous population of fused hybrid cells, *i.e.*, a hybridoma cell. Hybrid cells are cloned to establish cells lines producing a specific monoclonal antibody that is chemically and immunologically homogenous, *i.e.*, that recognizes only one type of antigen.

25        "Peptide linkage" refers to two or more amino acids covalently joined by a substituted amide linkage between the alpha-amino group of one amino acid and the alpha-carboxyl group of another amino acid.

"Pharmaceutically acceptable" refers to a non-toxic composition that is physiologically compatible with humans or other animals.

A "pharmaceutically acceptable excipient" comprises a material such as an adjuvant, a carrier, pH-adjusting and buffering agents, tonicity adjusting agents, wetting agents, preservatives, and the like, which is non-toxic and physiologically compatible with humans or other animals

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including *e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (*e.g.*, acridine, psoralen, etc.), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (*e.g.*, alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

15        "Protease cleavage site" refers to a site where proteolytic enzymes hydrolize (break) polypeptide chains.

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a compound of the invention will hybridize to its target sequence, but to a minimal number of other sequences.

20        The term "subject" refers to any warm-blooded animal, preferably a human.

"Tag" refers to a marker or marker sequence used to isolate or purify a molecule containing the tag. An exemplary tag includes a 6xHis tag.

"T cell" refers to a type of lymphocyte responsible for antigen-specific cellular interactions, and which mediates humoral and cellular immune responses.

25        "Target Binding Domain (TBD)" refers to a protein comprising an antibody fragment, which is capable of binding to a receptor on an antigen presenting cell, particularly on a dendritic cell, and which is subsequently transported into the antigen presenting cell by receptor-mediated uptake.

30        The phrase "therapeutically effective amount" refers to an amount of chimeric antigen, or polynucleotide encoding a chimeric antigen, sufficient to elicit an effective B cell, cytotoxic T lymphocyte (CTL) and/or helper T lymphocyte (Th) response to the antigen and to block or to cure or at least partially arrest or slow symptoms and/or complications of a disease or disorder.

The terms "treating" and "treatment" as used herein cover any treatment of a condition treatable by a chimeric antigen in an animal, particularly a human, and include: (i) preventing the condition from occurring in a subject which may be predisposed to the condition but has not yet been diagnosed as having it; (ii) inhibiting the condition, *e.g.*, arresting or slowing its development; or (iii) relieving the condition, *e.g.*, causing regression of the condition or its symptoms

"Xenotypic," as used herein, refers to originating from a different species other than the host. For example, a recombinantly expressed antibody cloned from a mouse genome would be xenotypic to a human but not to a mouse, regardless of whether that recombinantly expressed antibody was produced in a bacterial, insect or mouse cell.

### C. Novel chimeric Antigens

The invention provides chimeric antigens for eliciting an immune response comprising an immune response domain and a target binding domain, wherein the target binding domain comprises an antibody fragment. In accordance with the present invention, the chimeric antigen, preferably, is capable of binding to a Fc receptor and/or to a macrophage mannose receptor. The antibody fragment can be xenotypic to the host or not xenotypic to the host.

In preferred embodiments of the invention, the chimeric antigen is capable of inducing humoral and/or cellular immune responses. The cellular immune response can include a Th1 response, a Th2 response, and/or a cytotoxic T lymphocyte (CTL) response. In yet another preferred embodiment, the chimeric antigen elicits a multi-epitopic immune response. The multi-epitopic immune response can include a response to at least one epitope of the immune response domain and/or a response to at least one epitope of the target binding domain. Alternatively, the multi-epitopic response may be limited to a response to more than one epitope of the immune response domain.

The chimeric antigen of the present invention comprises two portions, namely an immune response domain containing an antigenic sequence (such as a viral antigen), and a target binding domain containing an antibody fragment (FIG.1). In a preferred embodiment, the immune response domain can be linked to the target binding domain by any method known to those of skill in the art. Linkers for linking the immune response domain to the target binding domain can include, but are not

limited to, covalent peptide linkages, chemical conjugation, leucine zippers and biotin/avidin. In a preferred embodiment, the immune response domain and target binding domain are cloned as a single fusion protein. The covalent peptide linkage of the fusion protein may comprise additional peptide sequences, such as SRPQGGGS or VRPQGGGS (SEQ ID NO: 1). In yet another preferred embodiment, various immune response domains are biotinylated and the target binding domain is generated with streptavidin as a fusion protein to facilitate the production of a wide assortment of chimeric antigens. Alternatively, the immune response domain and the target binding domain each can be expressed as a fusion to a leucine zipper moiety, which will cause the two portions of the chimeric antigen to associate upon mixing. Finally, the immune response domain and target binding domains can be expressed separately and then chemically conjugated using methods known to one of skill in the art. Exemplary methods include use of protein cross-linkers, such as dimethyl suberimidate, to covalently attach the two domains.

The immune response domain primarily provides the antigenic portion of the chimeric antigen. The immune response domain comprises at least one antigenic portion of the entity to which an immune response is desired. The chimeric antigen, optionally, can comprise more than one immune response domain. In preferred embodiments, the immune response domain comprises at least one antigenic portion of an infectious agent, such as a virus or an obligate intracellular parasite, or of a cancer antigen. More preferably, the immune response domain comprises at least one antigenic portion of an infectious virus.

Examples of preferred infectious viruses include: Retroviridae (*e.g.*, human immunodeficiency viruses, such as Human Immunodeficiency Virus-1 (HIV-1), also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (*e.g.*, polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (*e.g.*, strains that cause gastroenteritis); Togaviridae (*e.g.*, equine encephalitis viruses, rubella viruses); Flaviridae (*e.g.*, Hepatitis C virus, dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (*e.g.*, coronaviruses); Rhabdoviridae (*e.g.*, vesicular stomatitis viruses, rabies viruses); Filoviridae (*e.g.*, Ebola viruses); Paramyxoviridae (*e.g.*, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (*e.g.*, influenza viruses); Bungaviridae (*e.g.*, Hantaan viruses,

bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever virus); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (human Hepatitis B virus (HBV), duck Hepatitis B virus (DHBV)); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses);

5 Adenoviridae (most adenoviruses); Herperviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), Epstein-Barr viruses, herpes viruses); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g., African swine fever virus); and unclassified viruses (e.g., the agent of delta

10 hepatitis, the agents of non-A, non-B hepatitis (class 1--internally transmitted; class 2--parenterally transmitted; Norwalk and related viruses, and astroviruses). In some embodiments of the invention, the immune response domain of the chimeric antigen includes at least one antigenic portion of one or more proteins selected from the group consisting of HBV proteins, DHBV proteins, and HCV proteins.

15 } Particularly preferred HBV proteins for use in the present invention include, but are not limited to, HBV S1/S2, HBV S1/S2/S, HBV Core, HBV Core ctm (C-terminal modified), HBV e-antigen, and HBV polymerase. Particularly preferred DHBV proteins for use in the present invention include, but are not limited to, DHBV PreS/S, DHBV PreS, DHBV Core, and DHBV polymerase. Particularly preferred HCV proteins for use in the present invention include, but are not limited to, HCV Core (1-

20 191), HCV Core (1-177), HCV E1-E2, HCV E1, HCV E2, HCV NS3, HCV NS5A and NS4A. Other preferred viral antigens for use in the present invention include HIV gp120, HSV alkaline nuclease and human papilloma virus (HPV) capsid proteins L1 and L2, and early region proteins HPV E1, HPV E2, HPV E4, HPV E5, HPV E6, and HPV E7.

25 Examples of preferred obligate intracellular parasites include: *Tetrahymena* sp. (e.g. *T. pyriformis*), *Plasmodium* sp. (e.g. *P. falciparum*), *Cryptosporidium* sp., *Spraguea* sp. (e.g. *S. lophii*), *Giardia* sp., *Toxoplasma* sp. (e.g. *T. gondii*, *T. cruzi*), *Leishmania* sp., *Rickettsia* sp. (e.g. *R. prowazekii*), *Chlamydia* sp., *Mycobacterium* sp. (e.g. *M. tuberculosis*), *Legionella* sp., *Listeria* sp., (e.g. *L. monocytogenes*), *Coxiella*

30 sp. (e.g. *C. brunette*), *Shigella* sp., *Ehrlichia* sp., and *Bartonella* sp. Preferred cancer antigens include: prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), MUC1, CA 125, WT1, Her-2/neu, carcinoembryonic antigen (CEA), MAGE-3, MART-1, gp100, NY-ESO-1, CA19.9, TAG72, CA 15.3, CA 27.9, gp

120, prostatic acid phosphatase (PAP), Heatshock proteins, alpha-fetoprotein (AFP), Telomerase, and ras.

In yet another embodiment of the invention, the immune response domain of the chimeric antigen includes 6xHis tag fused to the one or more antigenic portions.

5 In accordance with the present invention, the chimeric antigen is a protein capable of binding to an Fc receptor and/or CD206 on an antigen presenting cell, particularly a dendritic cell, and is subsequently transported into the antigen presenting cell by receptor-mediated uptake. In accordance with the present invention, the presence of an antibody fragment augments the uptake of the chimeric antigen  
10 through the Fc receptor on antigen-presenting cells, specifically dendritic cells. By virtue of this specific binding and internalization, the viral antigen is processed and presented as foreign. Thus, an immune response can be effectively elicited to a previously tolerated antigen. The target binding domain comprises an antibody fragment that can be xenotypic to the host or not xenotypic to the host. In a preferred  
15 embodiment of the invention, the antibody fragment comprises a murine Fc fragment. In a more preferred embodiment of the invention, the target binding domain comprises a Fc fragment, a hinge region, and a portion of the C<sub>H</sub>1 region, and the chimeric antigen comprises a peptide linker suitable for linking the target binding domain to the immune response domain. In another preferred embodiment, the target  
20 binding domain comprises an immunoglobulin heavy chain fragment, and optionally, further comprises a hinge region. In a particularly preferred embodiment, the heavy chain fragment comprises amino acids, VDKKI (SEQ ID NO: 2) of the C<sub>H</sub>1 domain and/or part or all of the C<sub>H</sub>2 and C<sub>H</sub>3 domains.

As discussed above, antigens that are bound and internalized by CD206 can be  
25 presented by both MHC Class I and Class II, thus eliciting both a cellular and humoral immune response. Accordingly, in a preferred embodiment, the chimeric antigen is glycosylated. The immune response domain and/or the target binding domain can be glycosylated. In a particularly preferred embodiment, the chimeric antigen is mannose glycosylated by either high mannose glycosylation or by pauci mannose  
30 glycosylation (Jarvis, *Virology* 310:1-7 (2003)).

#### D. Novel Methods of Utilizing Chimeric Antigens

The invention includes methods of eliciting an immune response comprising administering, to a subject, a composition comprising a chimeric antigen of the invention.

5 In order to provide efficient presentation of the antigens, the inventors have developed a novel chimeric antigen comprising an immune response domain and a target binding domain, wherein the target binding domain comprises an antibody fragment. While not being limited to a particular theory of the invention, this molecule, by virtue of the antibody fragment binds to specific receptors on the antigen  
10 presenting cells, and the viral antigen is processed and presented complexed with Major Histocompatibility Complex (MHC) Class I and Class II. Such processing and antigen presentation by MHC Class I elicits an increased response by cytotoxic T-lymphocytes, resulting in the elimination of any infectious agent associated with the antigen of the immune response domain. In addition, antigen presentation by MHC  
15 Class II molecules, elicits a humoral response that also aids in clearance of the antigen from the infected cell and/or from circulation.

The invention also includes methods of breaking tolerance comprising administering a chimeric antigen of the invention to a subject. In the presentation of antigens to elicit a cellular and/or humoral immune response by the use of chimeric  
20 antigens, the antigens, which were treated as "self" during a chronic infection, are recognized as "foreign." Accordingly, the host's immune system will mount a CTL response to eliminate infected cells. At the same time, antibodies elicited in response to the chimeric antigen will bind to the infectious agent and remove it from the circulation or block binding of the infectious agent to host cells. Accordingly, the  
25 present invention is designed to produce chimeric antigens that can induce a broad immune response in subjects who have chronic infections that are otherwise tolerated by the host immune system. In a preferred embodiment, the chimeric antigen breaks tolerance to an antigen in a subject who is chronically infected with an infectious agent, such as a virus or parasite, or who has a cancer. More preferably, the infectious  
30 agent is resident inside a host cell at some point during its life cycle.

In a preferred embodiment, the immunogenicity of the pre-selected antigen unrecognized or tolerated by the host immune system is increased due to the presence of the antibody fragment as well as by the presence of glycosylation introduced in an

eukaryotic, *e.g.* insect, cell expression system. Such a chimeric antigen, due to the presence of the antibody component and glycosylation, will bind to specific receptors present on various immune cell types including dendritic cells, macrophages, B cells and granulocytes.

5 Yet another aspect of the invention provides methods of activating antigen presenting cells comprising contacting the antigen presenting cells with a chimeric antigen of the invention. The invention also provides methods of enhancing antigen presentation in an antigen presenting cell comprising contacting the antigen presenting cell with a composition comprising a chimeric antigen of the invention.

10 The chimeric antigen can be contacted with the antigen presenting cells, preferably dendritic cells, *in vivo* or *ex vivo*. In a preferred embodiment, contacting the chimeric antigen with antigen presenting cells activates the antigen presenting cells and enhances antigen presentation of more than one epitope. This multi-epitopic response can include presentation of one or more epitopes of the immune response domain

15 and/or presentation of one or more epitopes of the target binding domain.

The invention also provides methods of treating an immune-treatable condition comprising administering, to a subject in need thereof, a therapeutically effective amount of a chimeric antigen of the invention. In a preferred embodiment, the immune-treatable condition is an infection or a cancer. The infection can be a

20 viral infection, a parasitic infection or a bacterial infection. Preferably, the infection will have a stage during which the infectious agent is found within a host cell. More preferably, the immune-treatable condition is a chronic viral infection. Most preferably, the immune-treatable condition is a chronic hepatitis B viral (HBV) infection or a chronic hepatitis C viral (HCV) infection. For the treatment of HBV,

25 the immune response domain preferably comprises at least one antigenic portion of a protein selected from the group consisting of a HBV Core protein, a HBV S protein, a HBV S1 protein, a HBV S2 protein, and combinations thereof. For the treatment of HCV, the immune response domain preferably comprises at least one antigenic portion of a protein selected from the group consisting of a HCV Core (1-191)

30 protein, a HCV Core (1-177) protein, a HCV E1 protein, a HCV E2 protein, a HCV E1-E2 protein, a HCV NS3A protein, a HCV NS5A protein, and combinations thereof.

In a preferred embodiment, administration of the chimeric antigen elicits a greater immune response than administration of the immune response domain alone. The amplitude of the immune response can be measured, for example, (i) by the amount of antigen-specific antibody present in the subject; (ii) by the amount of interferon- $\gamma$  secreted by T cells in response to being exposed to antigen presenting cell loaded with the chimeric antigen or immune response domain alone; or (iii) by the amount of antigen specific CD8<sup>+</sup> T cells elicited in response to being exposed to antigen presenting cell loaded with the chimeric antigen or immune response domain alone.

The chimeric antigen can be evaluated for its efficacy in generating an immune response by presenting the chimeric antigen to dendritic cells *ex vivo* or *in vivo*. The dendritic cells process and present the chimeric antigen to T-lymphocytes, which are evaluated for proliferation of T cells and for the production of interferon- $\gamma$  as markers of T cell response. Specifically, in the *ex vivo* situation, naive dendritic cells are isolated from peripheral blood. Activation of the T cells by the dendritic cells is evaluated by measuring markers, *e.g.* interferon- $\gamma$  levels, by a known procedure. See, *e.g.*, Berlyn, et al., *Clin. Immunol* 101(3):276-283 (2001). An increase in the percentage of T cells that secrete interferon- $\gamma$  by at least 50% predicts efficacy *in vivo*. In the case of the *in vivo* situation, the chimeric antigen is directly introduced parenterally in the host where available dendritic and other antigen-processing cells have the capacity to interact with antigens and to process them accordingly.

Additionally, the invention includes methods of vaccinating a subject against an infection comprising administering a chimeric antigen of the present invention to the subject. The subject can be prophylactically or therapeutically vaccinated. Preferably, the infection is a viral infection. The bifunctional nature of the molecule helps to target the antigen to antigen-presenting cells, *e.g.* dendritic cells, making it a unique approach in the therapy of chronic infectious diseases by specifically targeting the antigen presenting cells with the most effective stoichiometry of antigen to antibody. This is useful to the development of therapeutic vaccines to cure chronic viral infections such as Hepatitis B, Hepatitis C, Human Immunodeficiency Virus, Human Papilloma Virus and Herpes Simplex Virus, obligate intracellular parasites and may also be applicable to all autologous antigens in diseases such as cancer and autoimmune disorders. The administration of these fusion proteins can elicit a broad

immune response from the host, including both cellular and humoral responses. Thus, they can be used as therapeutic vaccines to treat subjects that are immune tolerant to an existing infection, in addition to being useful as prophylactic vaccines to immunize subjects at risk for developing a particular infection.

#### 5        **E. Methods of Making Chimeric Antigens**

One aspect of the invention provides methods for producing a chimeric antigen comprising (a) providing a microorganism or cell line, preferably a eukaryotic, more preferably, a non-mammalian microorganism or cell line that comprises a polynucleotide encoding a chimeric antigen; and (b) culturing said  
10 microorganism or cell line under conditions whereby the chimeric antigen is expressed. Preferably, the microorganism or cell line is a yeast, a plant cell line or an insect cell line. More preferably, the cell line is an insect cell line selected from the group consisting of Sf9, Sf21, Drosophila S2, and High Five™.

One embodiment of the present invention uses established recombinant DNA  
15 technology for producing the fusion proteins of selected antigen(s) and the target binding domain that are necessary in the practice of the invention. Fusion protein constructs are generated at the DNA level incorporating specific restriction enzyme sites, which are exploited in incorporating the desired DNA fragment into expression vectors, and used to express the desired fusion proteins in a heterologous expression  
20 system. As used herein, the term "vector" denotes plasmids that are capable of carrying the DNA, which encode the desired protein(s). Preferred plasmid vectors for use in the present invention include, but are not limited to, pFastBac HTa and the corresponding recombinant "bacmids" generated in DH10Bac™ *E. coli* (Invitrogen).

A gene encoding a target binding domain can be obtained from any antibody  
25 producing cell, for example a hybridoma producing a monoclonal antibody, via polymerase chain reaction (PCR). To facilitate later cloning steps, it is preferable to design the oligonucleotide primers to add unique restriction enzyme recognition sites. Similarly, the antigenic portions of the immune response domain can be obtained from any cell or virus RNA or DNA containing a gene encoding an antigenic portion  
30 of the desired target. Preferably, PCR is used to obtain DNA encoding the antigenic portions of the immune response domain and the PCR primers are designed to add unique restriction enzyme recognition sites to facilitate cloning. However, any recombinant DNA method may be used to obtain the DNA encoding the antigenic

portions of the immune response domain. The polynucleotides encoding the target binding domain and immune response domain can then be combined in a single construct using standard cloning techniques. Alternatively, the separate domains can be cloned with DNA encoding linkers, such as leucine zippers, streptavidin or biotinylation signals.

Preferably, a baculovirus system is used to express the chimeric antigen of the invention not only because large amounts of heterologous proteins are produced, but also because post-translational modifications, such as phosphorylation and glycosylation, of eukaryotic proteins occur within the infected insect cell. Since cloning directly in insect cells can be difficult, it is preferable to generate the polynucleotide encoding the chimeric antigen in a bacterial system and to transfer the final construct into a baculovirus/insect cell expression system. Transfer systems, *e.g.*, the Bac-To-Bac™ system (Invitrogen), are known to those of skill in the art. The Bac-to-Bac™ system utilizes site-specific transposition with the bacterial transposon Tn7 to transfer the gene of interest into a *E. coli*-insect cell shuttle vector (bacmid). The resulting recombinant bacmids are transfected into insect cells to generate baculoviruses that express recombinant proteins.

In order to produce baculoviruses, the bacmid is transfected into insect cells, such as Sf9 cells. Following transfection, the cells are incubated for a period of time sufficient to expand the baculoviral population. The medium containing baculovirus is collected and stored at 4° C in the dark. The transfection can be verified by checking for production of baculoviral DNA by subjecting the viral culture to PCR utilizing primers specific for the desired DNA insert. The expression of the heterologous protein in the cells can be verified by any method known in the art, *e.g.* SDS polyacrylamide gel electrophoresis (SDS-PAGE) or Western blotting.

Recombinant bacmids of standardized multiplicity of infection (MOI) are used to infect insect cells. Cells are seeded at a density of approximately  $3 \times 10^5$  cells/mL and incubated in suspension culture at 27.5°C with shaking until the cell density reached approximately  $2-3 \times 10^6$  cells/mL. Standardized amounts of the respective recombinant baculovirus are then added to the cells. The incubation temperature is 27.5°C and the appropriate infection period is standardized for individual protein expression. The cells are harvested by centrifugation and used for the purification of

the recombinant proteins. Unused portions of cells can be snap frozen in liquid nitrogen and stored at -70°C.

Chimeric antigens, preferably, are purified under denaturing conditions. Cells expressing chimeric antigens are lysed in a denaturing buffer, *e.g.*, a buffer containing 5 6 M guanidinium-HCl. Lysis can be increased by mechanical means, such as sonication. The lysate is centrifuged to remove unbroken cells and cell debris. The supernatant is then loaded on to a Ni-NTA Super Flow (Qiagen) bead column pre-equilibrated with lysis buffer. Following loading, the column is washed with a buffered denaturing solution, preferably containing 6 M guanidinium-HCl at 10 approximately pH 8. At this point the denaturant can be exchanged to, *e.g.*, 8 M urea in a buffered solution. The lysis, loading and wash buffers preferably contain a low concentration, *e.g.*, 1-40 mM imidazole. After buffer exchange, the column should be washed with buffer until the OD<sub>280</sub> drops to, for example, <0.1. The bound protein can be eluted with a buffer containing 8 M urea, and 250 mM imidazole, pH 8 15 (Elution Buffer). The fractions containing the protein are pooled and dialyzed at 4°C against multiple changes of low (*e.g.*, 100 mM) salt denaturing dialysis buffer, preferably containing 8 M urea. The dialyzed protein is then loaded onto an ion exchange column, such as DEAE (diethylaminoethyl). In a preferred embodiment, dithiothreitol (DTT) or other reducing agent is added to the protein prior to loading 20 onto the ion exchange column. The chimeric antigen will pass through a DEAE column. Therefore, the DEAE flowthrough is collected and dialyzed in a stepwise manner against buffers containing decreasing concentrations of denaturant. In an exemplary method, the protein is then dialyzed against buffered 4 M urea for at least 12 hours, then against buffered 2 M urea for at least 12 hours, then against buffered 1 25 M urea for at least 12 hours, then against buffered 0.5 M urea for at least 12 hours and finally dialyzed against buffer containing no denaturant for at least 12 hours, preferably followed by two additional periods of 12 hours dialysis against fresh buffer containing no denaturant. Purified, refolded proteins can be concentrated and characterized using standard biochemical techniques including, *e.g.*, SDS gel 30 electrophoresis, isoelectric focusing, or western blot analysis using antibodies against different domains of the expressed protein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and

immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See *e.g.*, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Ed., New York: Cold Spring Harbor Press, 1989; and Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (©1995, as  
5 Supplemented April 2004, Supplement 66).

#### F. Novel Polynucleotides

Another aspect of the invention provides polynucleotides encoding a chimeric antigen comprising a first polynucleotide portion encoding an immune response domain and a second polynucleotide portion encoding a target binding domain. The  
10 first and second polynucleotide portions may be located on the same or different nucleotide chains.

The invention provides polynucleotides corresponding or complementary to genes encoding chimeric antigens, mRNAs, and/or coding sequences, preferably in isolated form, including polynucleotides encoding chimeric antigen variant proteins;  
15 DNA, RNA, DNA/RNA hybrids, and related molecules, polynucleotides or oligonucleotides complementary or having at least a 90% homology to the genes encoding a chimeric antigen or mRNA sequences or parts thereof, and polynucleotides or oligonucleotides that hybridize to the genes encoding a chimeric antigen, mRNAs, or to chimeric antigen-encoding polynucleotides.

20 Additionally, the invention includes analogs of the genes encoding a chimeric antigen specifically disclosed herein. Analogs include, *e.g.*, mutants, that retain the ability to elicit an immune response, and preferably have a homology of at least 80%, more preferably 90%, and most preferably 95% to any of polynucleotides encoding a chimeric antigen, as specifically described by SEQ ID NOs: 26, 28, 30, 32, 34, 36, 38,  
25 40, 42, 44, 46, and 44. Typically, such analogs differ by only 1 to 15 codon changes. Examples include polypeptides with minor amino acid variations from the natural amino acid sequence of a viral antigen or of an antibody fragment, in particular, conservative amino acid replacements. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically-  
30 encoded amino acids are generally divided into four families: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine.

Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid will not have a major effect on biological activity. Polypeptide molecules having substantially the same amino acid sequence as any of the polypeptides disclosed in any one of SEQ ID NOs: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47 and 49 but possessing minor amino acid substitutions that do not substantially affect the ability of the chimeric antigens to elicit an immune response, are within the definition of a chimeric antigen having the sequence as set forth in SEQ ID NOs: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47 and 49, respectively. Derivatives include aggregative conjugates with other chimeric antigen molecules and covalent conjugates with unrelated chemical moieties. Covalent derivatives are prepared by linkage of functionalities to groups that are found in chimeric antigen amino acid chains or at the N- or C-terminal residues by means known in the art.

Amino acid abbreviations are provided in Table 1.

**TABLE 1: Amino Acid Abbreviations**

|               |     |   |
|---------------|-----|---|
| Alanine       | Ala | A |
| Arginine      | Arg | R |
| Asparagine    | Asn | N |
| Aspartate     | Asp | D |
| Cysteine      | Cys | C |
| Glutamate     | Glu | E |
| Glutamine     | Gln | Q |
| Glycine       | Gly | G |
| Histidine     | His | H |
| Isoleucine    | Ile | I |
| Leucine       | Leu | L |
| Lysine        | Lys | K |
| Methionine    | Met | M |
| Phenylalanine | Phe | F |
| Proline       | Pro | P |
| Serine        | Ser | S |
| Threonine     | Thr | T |
| Tryptophan    | Trp | W |
| Tyrosine      | Tyr | Y |
| Valine        | Val | V |

Conservative amino acid substitutions can be made in a protein without altering either the conformation or the function of the protein. Proteins of the invention can comprise 1 to 15 conservative substitutions. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine (A) and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pKs of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments (see, *e.g.* Biochemistry 4<sup>th</sup> Ed., Lubert Stryer ed. (W. H. Freeman and Co.), pages 18-23; Henikoff and Henikoff, *Proc Nat'l Acad Sci USA* 89:10915-10919 (1992); Lei et al., *J Biol Chem* 270(20):11882-6 (1995)).

The inventions also provides polynucleotides that hybridize, preferably under stringent conditions, to a polynucleotide encoding a chimeric antigen, as specifically described by SEQ ID NOs: 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, and 44. Stringency of hybridization reactions is readily determinable by one of ordinary skill in the art and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured nucleic acid sequences to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature that can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see, *e.g.*, Ausubel et al., *supra*, at pages 2.9.1-2.10.8 and 4.9.1-4.9.13.

"Stringent conditions" or "high stringency conditions", as defined herein, are identified by, but not limited to, those that (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1X SSC containing EDTA at 55°C "Moderately stringent conditions" are described by, but not limited to, those in Sambrook et al., *supra*, and include the use of washing solution and hybridization conditions (*e.g.*, temperature, ionic strength and % SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 mg/mL denatured sheared salmon sperm DNA, followed by washing the filters in 1X SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

Embodiments of a chimeric antigen-encoding polynucleotide include: a polynucleotide encoding a chimeric antigen having a sequence selected from any of SEQ ID NOs: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45 47 and 49, or a nucleotide sequence of chimeric antigen selected from any of SEQ ID NOs: 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 and 48, wherein T, optionally, can be U; For example, embodiments of chimeric antigen nucleotides comprise, without limitation:

(a) a polynucleotide comprising or consisting of a sequence as described by SEQ ID NOs.: nucleotides 1 to 1326 of SEQ ID NO: 26, nucleotides 1 to 2004 of SEQ ID NO: 28, nucleotides 1 to 1350 of SEQ ID NO: 30; nucleotides 1 to 1293 of SEQ ID NO: 32, nucleotides 1 to 1794 of SEQ ID NO: 34, nucleotides 1 to 1581 of SEQ ID NO: 36, nucleotides 1 to 1389 of

SEQ ID NO: 38, nucleotides 1 to 1347 of SEQ ID NO: 40, nucleotides 1 to 2157 of SEQ ID NO: 42, nucleotides 1 to 1395 of SEQ ID NO: 44, nucleotides 1 to 1905 of SEQ ID NO: 46, or nucleotides 1 to 2484 of SEQ ID NO: 48, wherein T can also be U;

5 (b) a polynucleotide whose sequence is at least 80% homologous to a sequence as described by SEQ ID NOs: 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 or 48;

(c) a polynucleotide that encodes a chimeric antigen whose sequence is encoded by a DNA contained in one of the plasmids designated  
 10 pFastBacHTa HBV S1/S2-TBD, pFastBacHTa HBV core-TBD, pFastBacHTa HCV core(1-177)-TBD, pFastBacHTa HCV NS5A-TBD, and pFastBacHTa HCV E2-TBD deposited with the International Depository Authority of Canada (Bureau of Microbiology at Health Canada) as Accession Nos. 080504-03, 080504-04, 080504-05, 080504-02  
 15 and 080504-01 respectively;

(d) a polynucleotide that encodes a chimeric antigen whose sequence is amino acids 1 to 442 of SEQ ID NO: 27, amino acids 1 to 668 of SEQ ID NO: 29, amino acids 1 to 450 of SEQ ID NO: 31, amino acids 1 to 431 of SEQ ID NO: 33, amino acids 1 to 598 of SEQ ID NO: 35, amino acids 1 to 527  
 20 of SEQ ID NO: 37, amino acids 1 to 463 of SEQ ID NO: 39, amino acids 1 to 449 of SEQ ID NO: 41, amino acids 1 to 719 of SEQ ID NO: 43, amino acids 1 to 465 of SEQ ID NO: 45, amino acids 1 to 635 of SEQ ID NO: 47, or amino acids 1 to 828 of SEQ ID NO: 49;

(e) a polynucleotide that encodes a chimeric antigen-related protein that is at least 90% identical to an entire amino acid sequence described by amino  
 25 acids 1 to 442 of SEQ ID NO: 27, amino acids 1 to 668 of SEQ ID NO: 29, amino acids 1 to 450 of SEQ ID NO: 31, amino acids 1 to 431 of SEQ ID NO: 33, amino acids 1 to 598 of SEQ ID NO: 35, amino acids 1 to 527 of SEQ ID NO: 37, amino acids 1 to 463 of SEQ ID NO: 39, amino acids  
 30 1 to 449 of SEQ ID NO: 41, amino acids 1 to 719 of SEQ ID NO: 43, amino acids 1 to 465 of SEQ ID NO: 45, amino acids 1 to 635 of SEQ ID NO: 47, or amino acids 1 to 828 of SEQ ID NO: 49;

(f) a polynucleotide that is fully complementary to a polynucleotide of any one of (a)-(d); and

(g) a polynucleotide that selectively hybridizes under stringent conditions to a polynucleotide of (a)-(f).

5       The invention also provides recombinant DNA or RNA molecules containing a chimeric antigen polynucleotide, an analog or homologue thereof, including but not limited to phages, plasmids, phagemids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), as well as various viral and non-viral vectors well known in the art, and cells transformed or transfected with such  
10 recombinant DNA or RNA molecules. Methods for generating such molecules are well known (see, for example, Sambrook et al., 1989, *supra*).

      The invention further provides a host-vector system comprising a recombinant DNA molecule containing a chimeric antigen polynucleotide, analog or homologue thereof within a suitable prokaryotic or eukaryotic host cell. Examples of suitable  
15 eukaryotic host cells include a yeast cell, a plant cell, or an animal cell, such as a mammalian cell or an insect cell (e.g., a baculovirus-infectible cell such as a Sf9, Sf21, Drosophila S2 or High Five™ cell). Examples of suitable mammalian cells include various prostate cancer cell lines such as DU145 and TsuPr1, other transfectable or transducible prostate cancer cell lines, primary cells (PrEC), as well  
20 as a number of mammalian cells routinely used for the expression of recombinant proteins (e.g., COS, CHO, 293, 293T cells). More particularly, a polynucleotide comprising the coding sequence of chimeric antigen or a fragment, analog or homolog thereof can be used to generate chimeric antigen thereof using any number of host-vector systems routinely used and widely known in the art.

25       A wide range of host-vector systems suitable for the expression of chimeric antigens thereof are available, see for example, Sambrook et al., 1989, *supra*; Ausubel, *supra*, at pages 1.0.1-1.16.16, 9.01-9.17.3, and 13.4.1-13.6.5). Preferred vectors for insect cell expression include but are not limited to pFastBac HTa (Invitrogen). Using such expression vectors, chimeric antigens can be expressed in  
30 several insect cell lines, including for example Sf9, Sf21, Drosophila S2, and High Five. Alternatively, preferred yeast expression systems include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, and *Pichia august*. The

host-vector systems of the invention are useful for the production of a chimeric antigen.

A chimeric antigen or an analog or homolog thereof can be produced by cells transfected with a construct encoding a chimeric antigen. For example, Sf9 cells can be transfected with an expression plasmid encoding a chimeric antigen or analog or homolog thereof, the chimeric antigen is expressed in the Sf9 cells, and the chimeric antigen is isolated using standard purification methods. Various other expression systems well known in the art can also be employed. Expression constructs encoding a leader peptide joined in frame to the chimeric antigen coding sequence can be used for the generation of a secreted form of chimeric antigen.

As discussed herein, redundancy in the genetic code permits variation in chimeric antigen gene sequences. In particular, it is known in the art that specific host species often have specific codon preferences, and thus one can adapt the disclosed sequence as preferred for a desired host. For example, preferred analog codon sequences typically have rare codons (*i.e.*, codons having a usage frequency of less than about 20% in known sequences of the desired host) replaced with higher frequency codons. Codon preferences for a specific species are calculated, for example, by utilizing codon usage tables available on the INTERNET such as at world wide web URL [www.kazusa.or.jp/codon](http://www.kazusa.or.jp/codon).

Additional sequence modifications are known to enhance protein expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon/intron splice site signals, transposon-like repeats, and/or other such well-characterized sequences that are deleterious to gene expression. The GC content of the sequence is adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. Where possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures. Other useful modifications include the addition of a translational initiation consensus sequence at the start of the open reading frame, as described in Kozak, *Mol. Cell Biol.*, 9:5073-5080 (1989). Skilled artisans understand that the general rule that eukaryotic ribosomes initiate translation exclusively at the 5' proximal AUG codon is abrogated only under rare conditions (see, *e.g.*, Kozak *Proc Nat'l Acad Sci USA* 92(7): 2662-2666 (1995) and Kozak *Nucl Acids Res* 15(20): 8125-8148 (1987)).

### G. Pharmaceutical Compositions of the Invention

One aspect of the invention relates to pharmaceutical compositions comprising a pharmaceutically acceptable excipient and a chimeric antigen comprising an immune response domain and a target binding domain, wherein the target binding domain comprises an antibody fragment. In therapeutic applications, the pharmaceutical compositions can be administered to a subject in an amount sufficient to elicit an effective B cell, cytotoxic T lymphocyte (CTL) and/or helper T lymphocyte (Th) response to the antigen and to block infection or to cure or at least partially arrest or slow symptoms and/or complications or a disease or disorder. Amounts effective for this use will depend on, *e.g.*, the particular composition administered, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the subject, and the judgment of the prescribing physician.

The dosage for an initial therapeutic immunization (with chimeric antigen) generally occurs in a unit dosage range where the lower value is about 1, 5, 50, 500, or 1,000 ng and the higher value is about 10,000; 20,000; 30,000; or 50,000 µg. Dosage values for a human typically range from about 500 ng to about 50,000 µg per 70 kilogram subject. Boosting dosages of between about 1.0 ng to about 50,000 µg of chimeric antigen pursuant to a boosting regimen over weeks to months may be administered depending upon the subject's response and condition. Administration should continue until at least clinical symptoms or laboratory tests indicate that the condition has been prevented, arrested, slowed or eliminated and for a period thereafter. The dosages, routes of administration, and dose schedules are adjusted in accordance with methodologies known in the art.

A human unit dose form of a chimeric antigen is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable carrier, in one embodiment an aqueous carrier, and is administered in a volume/quantity that is known by those of skill in the art to be useful for administration of such polypeptides to humans (see, *e.g.*, Remington: The Science and Practice of Pharmacy, 20<sup>th</sup> Edition, A. Gennaro, Editor, Lippincott Williams & Wilkins, Baltimore, Md., 2000). As appreciated by those of skill in the art, various factors can influence the ideal dose in a particular case. Such factors include, for example, half life of the chimeric antigen, the binding affinity of the chimeric antigen,

the immunogenicity of the composition, the desired steady-state concentration level, route of administration, frequency of treatment, and the influence of other agents used in combination with the treatment method of the invention, as well as the health status of a particular subject.

5 In certain embodiments, the compositions of the present invention are employed in serious disease states, that is, life-threatening or potentially life-threatening situations. In such cases, as a result of the relative nontoxic nature of the chimeric antigen in preferred compositions of the invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these  
10 chimeric antigens relative to these stated dosage amounts.

The concentration of chimeric antigen of the invention in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of  
15 administration selected.

The pharmaceutical compositions can be delivered via any route known in the art, such as parenterally, intrathecally, intravascularly, intravenously, intramuscularly, transdermally, intradermally, subcutaneously, intranasally, topically, orally, rectally, vaginally, pulmonarily or intraperitoneally. Preferably, the composition is delivered by  
20 parenteral routes, such as subcutaneous or intradermal administration.

The pharmaceutical compositions can be prepared by mixing the desired chimeric antigens with an appropriate vehicle suitable for the intended route of administration. In making the pharmaceutical compositions of this invention, the chimeric antigen is usually mixed with an excipient, diluted by an excipient or  
25 enclosed within a carrier that can be in the form of a capsule, sachet, paper or other container. When the pharmaceutically acceptable excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the therapeutic agent. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups,  
30 aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the chimeric antigen, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

Some examples of suitable excipients include, but are not limited to, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup, and methyl cellulose. The formulations can additionally  
5 include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring agents. The compositions of the invention can be formulated so as to provide quick, sustained or delayed release of the chimeric antigen after administration to the subject by employing procedures  
10 known in the art. See, *e.g.*, Remington, *supra*, at pages 903-92 and pages 1015-1050.

For preparing solid compositions such as tablets, the chimeric antigen is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a chimeric antigen of the present invention. When referring to these preformulation compositions as homogeneous, it is meant that  
15 the chimeric antigen is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules.

The tablets or pills of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action.  
20 For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer, which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or  
25 coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored  
30 emulsions with edible oils such as corn oil, cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

In preparing a composition for parenteral administration strict attention must be paid to tonicity adjustment to reduce irritation. A reconstitutable composition is a

sterile solid packaged in a dry form. A reconstitutable composition is preferred because it is more stable when stored as a dry solid rather than in a solution ready for immediate administration. The dry solid is usually packaged in a sterile container with a butyl rubber closure to ensure the solid is kept at an optimal moisture range. A  
5 reconstitutable dry solid is formed by dry fill, spray drying, or freeze-drying methods. Descriptions of these methods may be found, *e.g.*, in Remington, *supra*, at pages 681-685 and 802-803.

Compositions for parenteral injection are generally dilute, and the component present in the higher proportion is the vehicle. The vehicle normally has no  
10 therapeutic activity and is nontoxic, but presents the chimeric antigen to the body tissues in a form appropriate for absorption. Absorption normally will occur most rapidly and completely when the chimeric antigen is presented as an aqueous solution. However, modification of the vehicle with water-miscible liquids or substitution with water-immiscible liquids can affect the rate of absorption. Preferably, the vehicle of  
15 greatest value for this composition is isotonic saline. In preparing the compositions that are suitable for injection, one can use aqueous vehicles, water-miscible vehicles, and nonaqueous vehicles

Additional substances may be included in the injectable compositions of this invention to improve or safeguard the quality of the composition. Thus, an added  
20 substance may affect solubility, provide for subject comfort, enhance the chemical stability, or protect the preparation against the growth of microorganisms. Thus, the composition may include an appropriate solubilizer, substances to act as antioxidants, and substances that act as a preservative to prevent the growth of microorganisms. These substances will be present in an amount that is appropriate for their function,  
25 but will not adversely affect the action of the composition. Examples of appropriate antimicrobial agents include thimerosal, benzethonium chloride, benzalkonium chloride, phenol, methyl p-hydroxybenzoate, and propyl p-hydroxybenzoate. Appropriate antioxidants may be found in Remington, *supra*, at p. 1015-1017.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid  
30 particles, vesicles, and the like, are used for the administration of the chimeric antigens of the present invention. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively,

compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

Compositions administered via liposomes may also serve: 1) to target the chimeric antigen to a particular tissue, such as lymphoid tissue; 2) to target selectively to antigen presenting cells; or, 3) to increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations, the chimeric antigen to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule that binds to a receptor prevalent among lymphoid cells, such as monoclonal antibodies that bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired chimeric antigen of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the chimeric antigens. Liposomes for use in accordance with the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka, et al., *Ann. Rev. Biophys. Bioeng.* 9:467-508 (1980), and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369. A liposome suspension containing a chimeric antigen may be administered intravenously, locally, topically, etc. in a dose which varies according to, *inter alia*, the manner of administration, the chimeric antigen being delivered, and the stage of the disease being treated.

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described herein. The compositions can be administered by the oral or nasal respiratory route for local or systemic effect. Compositions in pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be inhaled directly from the nebulizing device or the nebulizing device may be attached to a facemask tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be

administered, preferably orally or nasally, from devices that deliver the formulation in an appropriate manner.

Another formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the chimeric antigen of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art. See, for example, U.S. Pat. No. 5,023,252, herein incorporated by reference. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents.

Additionally, it may be advantageous to include at least one antiviral therapeutic or chemotherapeutic in addition to the chimeric antigen and pharmaceutical excipient. Antiviral therapeutics include, but are not limited to, peptidomimetics (such as amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir), polynucleotides (such as amplitgen and fomivirsen), purine/pyrimidinones (such as abacavir, acyclovir, adefovir, cidofovir, cytarabine, didanosine, dideoxyadenosine, dipivoxil, edoxudine, emtricitabine, entecovir, famciclovir, ganciclovir, idoxuridine, inosine pranobex, lamivudine, MADU, penciclovir, sorivudine, stavudine, tenofovir, trifluridine, valacyclovir, valganciclovir, vidarabine, zalcitabine, and zidovudine), sialic acid analogs (such as oseltamivir and zanamivir), acemannan, acetyl-leucine monoethanolamine, amantadine, amidinomycin, ateviridine, capravirine, delavirdine, *n*-docosanol, efavirenz, foscarnet sodium, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , kethoxal, lysozyme, methisazone, moroxydine, nevirapine, pentafuside, pleconaril, podophyllotoxin, ribavirin, rimantidine, stallimycin, statolon, termacamra, and traomantadine. Other appropriate antiviral agents are discussed in Remington: *supra*, at Chapter 87: Anti-Infectives, pp. 1507-1561, particularly pp. 1555-1560. Preferred antiviral therapeutics for inclusion in the pharmaceutical compositions of the present invention include adefovir, dipivoxil, entecovir, lamivudine and ribavirin.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes B-lymphocytes or T lymphocytes. Lipids have been identified as agents capable of priming CTL *in vivo*. For example, palmitic acid residues can be attached to the  $\epsilon$ - and  $\alpha$ -amino groups of a

lysine residue and then linked, *e.g.*, via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be administered either directly in a micelle or particle, incorporated into a liposome, or emulsified in an adjuvant, *e.g.*, incomplete Freund's adjuvant. In a  
5 preferred embodiment, a particularly effective immunogenic composition comprises palmitic acid attached to  $\epsilon$ - and  $\alpha$ -amino groups of Lys, which is attached via linkage, *e.g.*, Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine (P<sub>3</sub>CSS) can be used to prime  
10 virus specific CTL when covalently attached to an appropriate peptide (see, *e.g.*, Deres, et al., *Nature* 342:561 (1989)). Chimeric antigens of the invention can be coupled to P<sub>3</sub>CSS, for example, and the lipopeptide administered to an individual to specifically prime an immune response to the target antigen.

While the compositions of the present invention should not require the use of  
15 adjuvants, adjuvant can be used. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, detergents, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol,  
20 immunostimulatory polynucleotide sequences, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Additional adjuvants are also well known in the art.

## H. Article of Manufacture

25 Another aspect of this invention provides an article of manufacture that comprises a container holding a composition, comprising a chimeric antigen, that is suitable for injection or reconstitution for injection in combination with printed labeling instructions providing a discussion of how to administer the composition parenterally, *e.g.* subcutaneously, intramuscularly, intradermally, nasally or  
30 intravascularly. The composition will be contained in any suitable container that will not significantly interact with the composition and will be labeled with the appropriate labeling that indicates it will be for parenteral use. Associated with the container will be the labeling instructions consistent with the method of treatment as described

hereinbefore. The container that holds the composition of this invention may be a container having a liquid composition suitable for injection that has an appropriate needle for injection and a syringe so that the patient, doctor, nurse, or other practitioner can administer the chimeric antigen. Alternatively, the composition may be a dry or concentrated composition containing a soluble version of the chimeric antigen, to be combined or diluted with an aqueous or nonaqueous vehicle to dissolve or suspend the composition. Alternatively, the container may have a suspension in a liquid or may be an insoluble version of the salt for combination with a vehicle in which the insoluble version will be suspended. Appropriate containers are discussed in Remington, *supra*, pages 788-789, 805, 850-851 and 1005-1014

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use. A label can be present on the container to indicate that the composition is used for a specific therapy or non-therapeutic application, and can also indicate directions for either *in vivo* or *ex vivo* use, such as those described above. Directions and or other information can also be included on an insert which is included with the kit.

## V. EXAMPLES

The following non-limiting examples provide further illustration of the invention.

### A. Example 1: Construction of TBD Expression Vector

Mouse IgG1 DNA sequences encoding amino acids of a portion of C<sub>H</sub>1-Hinge-C<sub>H</sub>2-C<sub>H</sub>3 region was generated from mRNA isolated from the hybridoma (2C12), which produces mAb against HBV surface antigen (sAg). Total mRNA was isolated using Trizol® reagent (Gibco BRL cat. No. 15596-026) and the cDNA of the target binding domain (TBD; mouse immunoglobulins fragment) was generated by RT-PCR using Superscript First-strand Synthesis (Invitrogen Cat. No. 11904-018). The PCR primers contained linker sequences encoding the linker peptide -SRPQGGGS- (SEQ ID NO: 1) at the 5' terminus, a unique Not I site at the 5' and a unique Hind III restriction site at the 3' end. The resulting cDNA contains (5' Not I)-linker sequence-C<sub>H</sub>1(VDKKI) (SEQ ID NO: 2). -Hinge Region- C<sub>H</sub>2- C<sub>H</sub>3-(3' Hind

III). Following digestion with the respective enzymes, the fragment is ligated with pFastBac HTa expression vector plasmid (Invitrogen) using the same restriction enzyme sites. The 5' primer used for PCR amplification was (Sense) 5' TGTCATTCTGCGGCCGCAAGGCGGCGGATCCGTGGACAAGAAAATTGTGC  
5 CCAGG (SEQ ID NO: 3) and the 3' primer was (antisense) 5' ACGAATCAAGCTTTGCAGCCCAGGAGAGTGGGAGAG (SEQ ID NO: 4), which contained the Not I and Hind III sites, respectively. The following is the protocol used for directional cloning. The generated fragment was digested with the respective enzymes, purified on agarose gel and cloned into the vector plasmid. The  
10 DNA sequence and the correctness of the ORF were verified by standard sequencing methods.

Following the cloning of the DNA encoding the target binding domain into the pFastBac HTa donor plasmid, the recombinant proteins was expressed using the Bac-to-Bac™ baculovirus expression system (Invitrogen). The cloned gene was  
15 transferred into a baculovirus shuttle vector via site-specific transposition in a strain of *E. coli*, DH10Bac. The DH10Bac cells contain the shuttle vector, which confers kanamycin resistance and a helper plasmid, which encodes the transposase and confers resistance to tetracycline. A 100 µl aliquot of competent DH10Bac cells was thawed on ice, the pFastBac HTa based plasmids were added and the mixture  
20 incubated on ice for 30 minutes. The mixture was heat shocked for 45 seconds at 42°C and then chilled on ice for 2 minutes. The mixture was then added to 900 µL of LB media and incubated for 4 hours at 37°C. The transformed cells were serially diluted with LB to 10<sup>-1</sup> and 10<sup>-2</sup> and 100 µl of each dilution was plated on LB agar plates supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml  
25 tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG and incubated for at least 36 hours at 37°C. The gentamicin resistance was conferred by the pFastBac HTa and the X-gal and IPTG (isopropylthio-β-D-galactoside) were used to differentiate between white colonies (recombinant plasmids) and blue colonies (non-recombinant). The white colonies were picked and inoculated into 2ml of LB supplemented with 50 µg/ml  
30 kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline and incubated overnight at 37°C, with shaking. A sterile loop was used to sample a small amount of the overnight culture and the sample was streaked onto a fresh LB agar plate supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline,

100 µg/ml X-gal, and 40 µg/ml IPTG and incubated for at least 36 hours at 37°C to confirm a white phenotype. Recombinant bacmids were isolated by standard protocols (Sambrook, *supra*), the DNA sample was dissolved in 40 µl of TE (10mM Tris-HCL pH 8, 1mM EDTA) and used for transfections.

5 In order to produce baculoviruses, the bacmid was transfected into Sf9 insect cells. Sf9 cells ( $9 \times 10^5$ ) were seeded into each well of a 6-well cell culture dish (35mm wells) in 2 ml of ESF 921 (Expression Systems) and allowed to attach for at least 1 hour at 27°C. Transfections were carried out using Cellfection® Reagent (Invitrogen, Cat. No. 10362-010) as per the protocols provided by the supplier of the  
10 Sf9 cells. Following transfection, the cells were incubated at 27°C for 72 hours. The medium containing baculovirus was collected and stored at 4°C in the dark.

The efficiency of the transfection was verified by checking for production of baculoviral DNA. The isolated baculovirus DNA was subjected to PCR to screen for the inserted gene encoding the TBD. The primers used were (sense) 5'  
15 TATTCGGATTATTCATACCG (SEQ ID NO: 5) and 3' (antisense) 5' CTCTACAAATGTGGTATGGC (SEQ ID NO: 6). Amplified products were run on an agarose gel (0.8%). The expression of the heterologous protein in the cells was verified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots using the 6xHis tag monoclonal antibody (Clonetech) as the probe.

20 Once production of baculovirus and the expression of protein were confirmed, the virus production was amplified to produce a concentrated stock of the baculovirus that carry the gene encoding the target binding domain. It is standard practice in the art to amplify the baculovirus at least two times, and in all protocols described herein this standard practice was adhered to. After the second round of amplification, the  
25 concentration of the generated baculovirus was quantified using a plaque assay according to the protocols described by the manufacturer of the kit (Invitrogen). The most appropriate concentration of the virus to infect High Five cells and the optimum time point for the production of the desired protein was established as well. Generally, for the expression of the TBD an MOI of 1 and a time period of 48 hours  
30 was used.

## **B. Example 2: Construction of Chimeric Antigen Expression Vectors**

The DNA encoding the desired viral antigen was generated from the template using PCR methodology using the 5' sense and 3' anti-sense primers indicated in

Table 2. The 5' end of the resulting amplified fragment contained the unique restriction site "5' enzy," and the 3' end contained the unique restriction site "3' enzy," each of which was used for ligations.

5

**TABLE 2: Construction of Chimeric Antigen Vectors**

| Viral antigen    | Sense primer  | Anti-sense primer | Template            | 5' enzy | 3' enzy |
|------------------|---------------|-------------------|---------------------|---------|---------|
| HBV S1/S2        | SEQ ID NO: 7  | SEQ ID NO: 8      | pRSETB HBV S1/S2    | Bam HI  | Not I   |
| HBV S1/S2/S      | SEQ ID NO: 9  | SEQ ID NO: 10     | pAlt HBV 991        | Nco I   | Not I   |
| HBV Core         | SEQ ID NO: 11 | SEQ ID NO: 12     | pAlt HBV 991        | Nco I   | Not I   |
| DHBV PreS/S      | SEQ ID NO: 5  | SEQ ID NO: 13     | pFastBac Hta PreS/S | Eco RI  | Not I   |
| DHBV PreS        | SEQ ID NO: 5  | SEQ ID NO: 14     | pFastBac HTa PreS/S | Eco RI  | Not I   |
| DHBV Core        | SEQ ID NO: 15 | SEQ ID NO: 16     | pRSETB DHBV Core    | Nco I   | Not I   |
| HCV Core (1-191) | SEQ ID NO: 17 | SEQ ID NO: 18     | pCV-H77c            | Eco RI  | Spe I   |
| HCV Core (1-177) | SEQ ID NO: 17 | SEQ ID NO: 19     | pCV-H77c            | Eco RI  | Spe I   |
| HCV NS5A         | SEQ ID NO: 20 | SEQ ID NO: 21     | pCV-H77c            | Eco RI  | Spe I   |
| HCV E1           | SEQ ID NO: 22 | SEQ ID NO: 23     | pCV-H77c            | Eco RI  | Spe I   |
| HCV E2           | SEQ ID NO: 24 | SEQ ID NO: 25     | pCV-H77c            | Eco RI  | Spe I   |
| HCV E1/E2        | SEQ ID NO: 22 | SEQ ID NO: 25     | pCV-H77c            | Eco RI  | Spe I   |

Amplified DNA was digested with appropriate 5' and 3' restriction enzymes and ligated with a pFastBac HTa expression vector to generate the expression plasmid for the viral antigen alone. The same fragment of DNA was also ligated with the plasmid pFastBac HTa-TBD, described in Example 1, following digestion with the respective enzymes to produce the expression plasmid for the viral antigen fused to the target binding domain. The resulting plasmid was used to produce recombinant baculovirus, as described in Example 1, for subsequent use in expression of the chimeric antigen. The DNA and amino acid sequences of the chimeric antigens are provided in Table 3.

**TABLE 3: Chimeric Antigen Sequences**

| Construct           | DNA sequence  | Expressed Protein |
|---------------------|---------------|-------------------|
| HBV S1/S2-TBD       | SEQ ID NO: 26 | SEQ ID NO: 27     |
| HBV S1/S2/S-TBD     | SEQ ID NO: 28 | SEQ ID NO: 29     |
| HBV Core-TBD        | SEQ ID NO: 30 | SEQ ID NO: 31     |
| DHBV PreS-TBD       | SEQ ID NO: 32 | SEQ ID NO: 33     |
| DHBV PreS/S-TBD     | SEQ ID NO: 34 | SEQ ID NO: 35     |
| DHBV Core-TBD       | SEQ ID NO: 36 | SEQ ID NO: 37     |
| HCV Core(1-191)-TBD | SEQ ID NO: 38 | SEQ ID NO: 39     |
| HCV Core(1-177)-TBD | SEQ ID NO: 40 | SEQ ID NO: 41     |
| HCV NS5A-TBD        | SEQ ID NO: 42 | SEQ ID NO: 43     |
| HCV E1-TBD          | SEQ ID NO: 44 | SEQ ID NO: 45     |
| HCV E2-TBD          | SEQ ID NO: 46 | SEQ ID NO: 47     |
| HCV E1/E2-TBD       | SEQ ID NO: 48 | SEQ ID NO: 49     |

### C. Example 3: Expression and Purification of TBD, Viral Antigens and Chimeric Antigens

5           Recombinant bacmids of standardized multiplicity of infection (MOI) were used to infect High Five™ insect cells. For suspension cultures, cells were seeded at a density of  $3 \times 10^5$  cells/mL and incubated at 27.5°C with shaking at 138 rpm until the cell density reached  $2-3 \times 10^6$  cells/mL. Standardized amounts of the respective recombinant baculovirus was added to the cells. The incubation temperature was 10 27.5°C and the appropriate infection period was standardized for individual protein expression. The cells were harvested by centrifugation at 2,500 rpm for 10 minutes at 4 °C and used for the purification of the recombinant proteins. Unused portions of cells were snap frozen in liquid nitrogen and stored at -70°C.

Recombinant proteins were purified under denaturing conditions. The cells 15 were lysed in a buffer containing 6 M Guanidinium-HCl in 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 300 mM NaCl, 10 mM Imidazole, pH 8.0 (lysis buffer). The suspension was sonicated on ice with 5 pulses of 1 minute per pulse at a power setting of 60 watts, and was mixed at room temperature for 1 hour. The lysate was centrifuged at 10,000 x g for 10 min to remove unbroken cells and cell debris. The supernatant was 20 loaded on to a Ni-NTA agarose (Qiagen) bead column (1 x 5 cm/100 mL cell lysate), pre-equilibrated with lysis buffer. Following loading, the column was washed with 20 column volumes of 6 M guanidinium-HCl in 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 300 mM NaCl, 40 mM Imidazole, pH 8.0 (wash buffer 1), followed by washes with 20

column volumes of 8 M urea in 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 300 mM NaCl, 40 mM imidazole, pH 8.0 (wash buffer 2). The bound protein was eluted with a buffer containing 8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8 (Elution Buffer). The fractions containing the protein were pooled and dialyzed at 4°C against multiple changes of dialysis buffer (10mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl). Purified proteins were characterized using standard biochemical techniques including SDS gel electrophoresis, isoelectric focusing, and western blot analysis using antibodies against different domains of the expressed protein.

#### 10        **D. Example 4: Breaking Tolerance to a “ Self ” Protein Using a Chimeric Antigen Fusion Protein.**

In order to evaluate the immune response to chimeric antigen fusion proteins, mice were immunized with purified HBV S1/S2, TBD or S1/S2-TBD proteins and the antibodies produced against the individual proteins were quantified. Proliferation of splenic T cells harvested from immunized mice was evaluated following challenge with the respective proteins.

BALB/c mice at 15 weeks of age were used for the immunizations. Mice were injected subcutaneously with S1/S2-TBD (4.15 µg), S1/S2 (4.15 µg) or TBD (4.15 µg) four times at two week intervals. Blood samples were collected before the start of immunizations and a week following each of the immunizations. Serum was prepared from clotted blood samples and used for the estimation of antibody levels produced by the host animal against the respective antigens injected.

##### 1. ELISA for detection of antibodies against HBV S1/S2, TBD or S1/S2-TBD

A 96-well plate was coated with antigens HBV S1/S2, TBD or S1/S2-TBD at a concentration of 1.0 µg/mL overnight at 4°C. The plate was washed with PBS containing 2% BSA. Diluted serum from the respective animal was added to each of the wells at various dilutions (1:10 - 1:500) and incubated at 37°C for 1hr. The plate was washed with PBS containing 0.05% Tween 20 (wash buffer). Goat anti-mouse IgG Fab-horse radish peroxidase (HRP) (1:5000) dilution was added to the wells and incubated at 37°C for 1hr. The plate was washed with wash buffer and color was developed using 2-2' azino-di-(3-ethyl benzylthiazoline-6-sulfonate) (KPL, Guildford,

UK). The optical density of resulting color in the samples was measured using an ELISA plate reader (Molecular Devices, USA) at a wavelength of 405 nm. Negative control for the experiment was pre-immune serum from the same animal, which was subtracted from all the experimental values. The results for mice immunized with HBV S1/S2-TBD are presented in Table 4. The chimeric antigen elicits a strong antibody response against the chimeric antigen (S1/S2-TBD).

**TABLE 4: Humoral response to S1/S2-TBD**

|                     |               | Level of antibody binding to: |           |       |
|---------------------|---------------|-------------------------------|-----------|-------|
|                     |               | HBV S1/S2-TBD                 | HBV S1/S2 | TBD   |
| Mice immunized with | HBV S1/S2-TBD | 0.192                         | 0.059     | 0.048 |
|                     | HBV S1/S2     | 0.073                         | 0.015     | 0.025 |
|                     | TBD           | 0.076                         | 0.017     | 0.036 |

The antibody response here is of a multivalent (or multi-epitopic) nature. The results presented in Table 4 show that antibodies produced by mice immunized with HBV S1/S2-TBD bind to the chimeric antigen and to the S1/S2 protein target coated on the plate. Therefore, antibodies are produced against the S1/S2 component of the chimeric antigen. Likewise, antibodies produced by mice immunized with HBV S1/S2-TBD bind to the target binding domain protein (Table 4). Chimeric antigen that contains protein of mouse origin can generate a humoral immune response in a mouse, evidence that the chimeric antigen can convert a “self” antigen into “foreign.” Accordingly, it is possible to break tolerance to a protein otherwise treated as a “self” protein.

## 2. T cell proliferation assay

Animals were sacrificed a week after the fourth immunization, the spleen was removed, and a single cell suspension was produced. Cells were seeded in triplicate at a cell density of  $4 \times 10^5$  cells/well in a 96-well plate. They were loaded with the respective antigens, HBV S1/S2, TBD or S1/S2-TBD, at concentrations of 0.1  $\mu\text{g/mL}$ , 1.0  $\mu\text{g/mL}$  and 10  $\mu\text{g/mL}$ . Negative control cells received media alone and the positive control for T cell proliferation was Phytohemagglutinin (PHA) at 1.0 – 5.0  $\mu\text{g/mL}$ . The cell cultures were incubated for 4 days at 37°C under an atmosphere of 7% CO<sub>2</sub>. Each of the wells of cells was pulsed with 1.0 mCi of <sup>3</sup>[H]-thymidine and incubated for an additional 18 hours. The cells were harvested using TOMTEC

MACH 3 cell harvester (Hamden, CT, USA) and the radioactivity bound to the glass fibre filter (Wallac Oy, Turku, Finland) was quantified using a Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (Wallac, USA). The results are shown in Table 5.

5

**TABLE 5: Cellular response to HBV S1/S2-TBD**

|               | Mean counts per minute (CPM) |            |             |
|---------------|------------------------------|------------|-------------|
|               | 0.1 µg/mL                    | 1 µg/mL    | 10 µg/mL    |
| HBV S1/S2-TBD | 36.7 ± 17.0                  | 32.7 ± 5.0 | 21.3 ± 7.0  |
| HBV S1/S2     | 10.7 ± 2.1                   | 26.7 ± 9.6 | 25.7 ± 10.3 |
| TBD           | 32.7 ± 19.1                  | 17.0 ± 2.6 | 35.7 ± 27.2 |
| Cells alone   | 21.3 ± 12.5                  |            |             |
| 1.0 mg/ml PHA | 39.3 ± 18.6                  |            |             |

T cell proliferation was seen when challenged with HBV S1/S2-TBD, S1/S2 or TBD. Immunization with the chimeric antigen induced a multivalent T cell response, *i.e.*, a response against different parts of the same protein. Chimeric antigen that contain protein of mouse origin can generate a cellular immune response in a mouse, evidence that the chimeric antigen can convert a “self” antigen into “foreign.” Therefore, it is possible to break tolerance to a protein otherwise treated as a “self” protein.

#### 15 **E. Example 5: Antigen Presentation Assays**

The ability of HBV S1/S2-TBD to elicit an immune response was measured using an *ex vivo* antigen presentation assay. The generation of an effective T cell response following multiple stimulations of naïve T cells with antigen-loaded antigen presenting cells (APCs) such as dendritic cells (DCs) was assessed by quantitating the increase in the number of antigen-specific T cells as well as the ability of the T cells to produce the Th1 cytokine IFN-γ.

##### **1. Selection of Monocytes by Adhesion**

Peripheral blood mononuclear cells (PBMCs) were thawed by the addition of AIM-V (ratio of 9 ml of AIM-V added to 1 ml of frozen cells). The cells were then centrifuged at 200x g for 5 min, the supernatant removed, and the cells resuspended in AIM-V/1% matched serum and added to either a 100 mm culture dish or a T-25 culture flask. The PBMCs were incubated for 1 hr at 37°C in a humidified incubator

25

under 7% CO<sub>2</sub>. To remove non-adherent cells, the culture was triturated several times, the supernatant discarded, and the cells washed once with AIM-V medium. Monocytes were harvested with a cell scraper and centrifuged at 300x g for 5 min. The cell pellet was re-suspended in AIM-V/2.5% matched serum at 2 x 10<sup>6</sup> cells/ml and seeded into a 24-well dish. The cytokines IL-4 and GM-CSF (1000 IU/ml each) were added to drive the differentiation of monocytes into immature DCs.

## 2. Fast or Slow Antigen Presentation Assay

For the Fast Antigen Presentation Assay (APA), antigen was added to immature DCs within 4 to 24 hr of isolation. After a further 24 hr, antigen loaded immature monocytes were induced to mature by culturing with PGE<sub>2</sub> (1 μM), IL-1β (10 ng/ml), and TNF-α (10 ng/ml) for 24 hr. The mature DCs were then co-cultured (first stimulation) with autologous T cells. The T cells were generated from the same PBMCs as the DCs by means of negative selection using a magnetic T cell isolation kit (Dynal) according to the manufacturer's directions.

T cells were then re-stimulated 7 days later with antigen loaded mature DCs in the presence of IL-2 (20 IU/ml), IL-7 (10 ng/ml), and IL-15 (5 ng/ml). Following a 7 day incubation, T cells were re-stimulated a third time with antigen loaded mature DCs. The third stimulation lasted for 6 hr whereupon the T cells were harvested and immunostained for CD3, CD8 and IFN-γ expression, and analyzed by flow cytometry.

For the Slow APA, monocytes were allowed to differentiate into immature DCs in the presence of GM-CSF and IL-4 for 5 to 6 days before addition of antigen. Two hours after antigen addition, immature DCs were matured with TNF-α (10 ng/ml) and IFN-α (50 IU/ml). Seven days post isolation, the mature DCs were co-cultured (first stimulation) with autologous T cells (described above).

T cells were then re-stimulated 7 days later with antigen loaded mature DCs in the presence of IL-2, IL-7, and IL-15. Following a 7 day incubation, cells were re-stimulated a third time with antigen loaded mature DCs. After an 18 hr incubation the T cells were harvested and immunostained for CD3, CD8 and IFN-γ expression, and analyzed by flow cytometry.

## 2. PBMC Antigen Presentation Assay

In this assay, the initial culture consists of total PBMCs (ie. lymphocytes and monocytes) that are incubated with antigen and IL-2 with the assumption that the

system resembles the *in vivo* immune response since all the cell types are present to participate (Maini, M.K et. al J. Exp. Med. 191:1269-1280, 2000). PBMCs are thawed, washed and immediately incubated with antigen. Following 4 days of culture to allow for antigen uptake and presentation, IL-2 (20 IU/ml) was added and left for an additional 8 days (i.e. day 12 of the experiment). Two days prior to the second stimulation (i.e. day 10 of the experiment), DCs are isolated by adhesion as described above, and immediately incubated with GM-CSF, IL-4 and antigen for 24 hr. As with the Fast APA, the immature DCs are allowed to differentiate for 24 hr following the addition of PGE<sub>2</sub>, IL-1 $\beta$ , and TNF- $\alpha$ . The loaded mature DCs are then added to the PBMC culture (second stimulation, day 12 of the experiment) in the presence of IL-2, IL-7, and IL-15. The third stimulation occurred on day 21 of the experiment with antigen loaded mature DCs prepared 2 days prior as described. Following a 6 hr incubation, the T cells were harvested and immunostained for CD3, CD8 and IFN- $\gamma$  expression, and analyzed by flow cytometry.

For all antigen presentation assays discussed above, a portion of the T cells at the end of the assay were incubated for an additional 3 – 5 days and examined for specific T cells by tetramer analysis (see below).

#### 4. HBV S1/S2 Elicits a T cell Response Against HBV S1 and S2

##### Peptides

The PBMC APA was used to generate T cells which were then assessed for their antigen specificity. Thus, PBMCs from healthy HLA-A2 individuals were cultured in AIM-V containing 2.5% matched sera in 96-well plates at  $5 \times 10^5$  cells/ml. Antigen (ie. 10  $\mu$ g/ml S1/S2-TBD) was added and the cells were cultured for 4 days at 37°C. IL-2 was then added at 20 IU/ml and the cells were cultured for an additional 8 days, with media changes (AIM-V/2.5% matched serum and 20 IU/ml IL-2) every 2-3 days. The majority of the cells remaining at the end of the 12 day culture were T cells, and these T cells were restimulated with autologous antigen-loaded mature DCs in the presence of IL-2 (20 IU/ml), IL-7 (10 ng/ml), and IL-15 (5 ng/ml).

The antigen-loaded mature DCs for the second and third stimulation of the T cells in the APA were generated over a 48 hr period using the procedure described below. The monocytes were isolated from total PBMC by adherence on plastic tissue culture dishes. The cells, about 85% of which were monocytes as determined by FACS analysis (CD11c+, CD14+, CD19-, and CD3-), were cultured in a 96-well plate

at  $1 \times 10^5$  cells/well containing 100  $\mu$ l of AIM-V/2.5% matched sera with the cytokines IL-4 and GM-CSF at 1000 IU/ml, and 4 hr later antigen such as S1/S2-TBD was added. Following a 20 hr incubation, the generated immature DCs were differentiated to mature DCs by culturing for a further 24 hr in the presence of PGE<sub>2</sub> (1  $\times 10^{-6}$  M), IL-1 $\beta$  (10 ng/ml), and TNF- $\alpha$  (10 ng/ml).

T cells were cultured for 7 days following the second stimulation, with media changes (AIM V with 2.5% matched serum and 20 IU/ml IL-2) every 1-2 days. The T cells (day 19 of culture) were then stimulated a third time with antigen-loaded mature DC (generated over a 2 day procedure as outlined above) in the presence of IL-2, IL-7, and IL-15 (as above) and either assessed for IFN- $\gamma$  production after a 6 hr culture or cultured for 5 days (with media changes every 1-2 days with AIM-V/2.5% matched serum and 20 IU/ml IL-2) and then assessed for T cell specificity to HBV preS antigen using HBV preS tetramers (day 24 of culture).

Tetramer analysis was performed with custom synthesized iTag MHC class I tetramers (Beckman Coulter) according to the manufacturer's protocol. Thus cells were harvested, washed, and transferred to a 96-well v-bottom plate at  $\sim 2 \times 10^5$  cells/well in 20  $\mu$ l. The cells were labeled at 20°C for 30 min with mAb specific to CD3 (anti-CD3-FITC) and CD8 (anti-CD8-Cy-Chrome) together with either 2  $\mu$ l PE-conjugated HLA-A\*0201 preS1 tetramer (GMLTPVSTI, SEQ ID NO: 50) or a preS2 tetramer (NIASHISSI, SEQ ID NO: 51). The cells were then washed, fixed with 2% paraformaldehyde in PBS and transferred into 5 ml FACS tubes. The cells were acquired on a FACSCalibur (BD Biosciences) with 80,000–100,000 events per sample. Analysis was performed using CellQuest software (BD Biosciences) with a gate on the viable (based on the FSC/SSC profile) CD3+ population and the percentage of CD8+ cells labeling with tetramer was determined. When PBMCs were cultured with HBV S1/S2-TBD at 10  $\mu$ g/ml and restimulated twice with HBV S1/S2-TBD-loaded mature DC, a marked percentage of the cells labeled positive with S1 tetramer (Fig. 2) and S2 tetramer (Fig. 3). This is in contrast to T cells cultured with antigen-unloaded mature DC, where the number of tetramer positive cells was not significant. Thus, S1/S2-TBD-loaded mature DC were able to induce the generation of a significant number of T cells with specificity to determinants of HBV S1 and HBV S2 antigens.

## **F. Example 6: Breaking Tolerance to DHBV and DHBV Antigens Using Chimeric Antigen Fusion Protein.**

DHBV has served as a powerful animal model in the development of antiviral therapy for HBV. Pekin ducks, congenitally infected with DHBV have been used to study the mechanism of replication of the virus and for the screening of antiviral compounds. Two kinds of duck models were used in the present invention. First is the congenitally DHBV-infected ducks. This resembles the vertical transmission of the HBV infection in man. The second model is the persistent infection model where newly hatched ducklings are infected with DHBV and these carry the infection. This second model resembles the horizontal transmission of the HBV infection in man.

### **1. Congenitally DHBV-infected ducks**

Congenitally DHBV-infected ducks, at four weeks of age, were divided into two groups. A sample of blood (1.0 mL) was collected for reference of pre-immunization antibody levels and blood samples were collected every week before the vaccinations. The experimental group received DHBV Core-TBD chimeric antigen fusion protein 19.95 mg/dose injected subcutaneously every week on the same day until week 5. During week 6, the dose was doubled and injected once every four weeks until vaccinations were discontinued at week 26. The placebo group received the equivalent volume of the buffer (20mM Sodium Phosphate pH 8.0, 300mM NaCl).

A 96-well plate was coated with antigens, DHBV Core, TBD or DHBV Core-TBD, at a concentration of 1.0 µg/mL overnight at 4°C. The plate was washed with phosphate buffered saline (PBS) containing 2% BSA. Diluted serum from the respective animal was added to each of the wells at various dilutions (1:10- 1:500) and incubated at 37°C for 1hr. The plate was washed with PBS containing 0.05% Tween 20 (wash buffer). Goat anti-duck IgG-HRP (1:5000) dilution was added to the wells and incubated at 37°C for 1hr. The plate was washed with wash buffer and color was developed using 2-2'-azino-di-(3-ethylbenzylthiazoline-6-sulfonate) (KPL, Guildford, UK). The optical density of resulting color in the samples was measured using an ELISA plate reader (Molecular Devices, USA). Antibody titers were calculated relative to the pre-immune serum from the same animal.

Anti-core antibody levels in the serum from congenitally DHBV-infected ducks in the control and experimental groups of ducks, at weeks 0, 3 and 6, are shown

in Table 6. Although the ducks have a chronic DHBV infection, the antibody levels are low, due to the chronic nature of the infection and the immune system not recognizing the antigen as a foreign molecule. On immunization with DHBV Core-TBD chimeric antigen, the host immune system recognized the viral antigen and mounted a humoral response against the core antigen that is already present in the host, thus breaking the host tolerance to a viral antigen.

**TABLE 6: Humoral response to DHBV Core-TBD**

|                  |        | Antibody binding (OD <sub>405</sub> ) |               |
|------------------|--------|---------------------------------------|---------------|
|                  |        | anti-DHBV Core                        | anti-TBD      |
| Vaccinated ducks | Week 0 | 0.058 ± 0.005                         | 0.005 ± 0.003 |
|                  | Week 3 | 0.131 ± 0.029                         | 0.092 ± 0.059 |
|                  | Week 6 | 0.166 ± 0.021                         | 0.147 ± 0.038 |
| Control Group    | Week 0 | 0.062 ± 0.016                         | 0.003 ± 0.002 |
|                  | Week 3 | 0.074 ± 0.015                         | 0.010 ± 0.005 |
|                  | Week 6 | 0.087 ± 0.012                         | 0.035 ± 0.017 |

Similarly, the duck immune system recognized the TBD component of the chimeric antigen as a foreign antigen and generated an immune response against this part of the fusion protein as well. Plates were coated with TBD and the serum from individual ducks evaluated for the antibody levels by ELISA. The results from this study are presented in Table 6.

## 2. Post-hatch DHBV-infected ducks

Normal ducklings were infected with DHBV-containing duck serum a day after the ducklings were hatched. This is standard practice in the field of DHBV research. The presence of persistent viremia was verified using established techniques at week four before the start of the immunizations. DHBV-infected ducks were divided into two groups. A sample of blood (1.0 mL) was collected from each duck for reference of pre-immunization antibody levels and blood samples were collected every week before the vaccinations. The experimental group received DHBV Core-TBD chimeric antigen fusion protein 19.95 µg/dose injected subcutaneously every week on the same day until week 5. During week 6, the dose was doubled and injected once every four weeks until vaccinations were discontinued at week 30. Blood samples were collected from the placebo group, which received the equivalent volume of the buffer (20mM Sodium Phosphate pH 8.0, 300mM NaCl).

Antibody levels in sera collected from ducks at weeks 0, 3 and 6 are presented. Anti-core antibody levels in the serum from post-hatch DHBV-infected ducks in the control and experimental groups of ducks are shown in Table 7. Since DHBV has established a persistent infection, the antibody levels are low, as the immune system does not recognize the viral antigen as a foreign molecule. On immunization with DHBV Core-TBD chimeric antigen, the host immune system recognized the viral antigen and mounted a humoral response against the core antigen that is already present in the host, thus breaking the host tolerance to a viral antigen. The antibody levels against TBD also increased (Table 7). Therefore there is a multivalent (or multi-epitopic) immune response against different parts of the same chimeric antigen.

**TABLE 7: Humoral response to DHBV Core-TBD**

|                  |        | Antibody binding to: |               |
|------------------|--------|----------------------|---------------|
|                  |        | DHBV Core            | TBD           |
| Vaccinated ducks | Week 0 | 0.066 ± 0.011        | 0.003 ± 0.002 |
|                  | Week 3 | 0.145 ± 0.014        | 0.072 ± 0.043 |
|                  | Week 6 | 0.170 ± 0.009        | 0.163 ± 0.052 |
| Control Group    | Week 0 | 0.083 ± 0.016        | 0.008 ± 0.010 |
|                  | Week 3 | 0.112 ± 0.042        | 0.011 ± 0.007 |
|                  | Week 6 | 0.138 ± 0.041        | 0.026 ± 0.013 |

#### **G. Example 7: Chemically Cross-Linked HBV sAg-Fc (Murine)**

Solutions of 100 µg sAg (US Biologicals; Cat# H 1910-27) and 100 µg Mouse Polyclonal IgG Fc fragment (Harlan Sera-Lab Ltd., Cat# PP-19-01) were dialyzed against 100 mM HEPES pH 8.7 overnight at 4°C. The protein solutions were mixed together, dimethyl suberimidate (DMS; Pierce Cat # 20700) was added immediately to a final concentration of 10 mM, and the mixture was incubated at room temperature for 1 hr. The reaction was stopped by the addition of 0.1 M Tris HCl pH 7.8. The reaction mixture was loaded on a Sephadex G 75 column (0.7 x 12 cm), and fractions were eluted using phosphate buffered saline. 0.5 ml fractions were collected and the fractions containing sAg/Fc at a molar ratio of 1:1, as estimated by ELISA using the respective antibodies were pooled.

The pooled fractions were used for antigen presentation assays. (Berlyn, et al., *Clin. Immunol.* 101: 276-283, (2001)). Immature dendritic cells were cultured for four days with GM-CSF/IL4, incubated with the sAg-Fc conjugate and matured in the

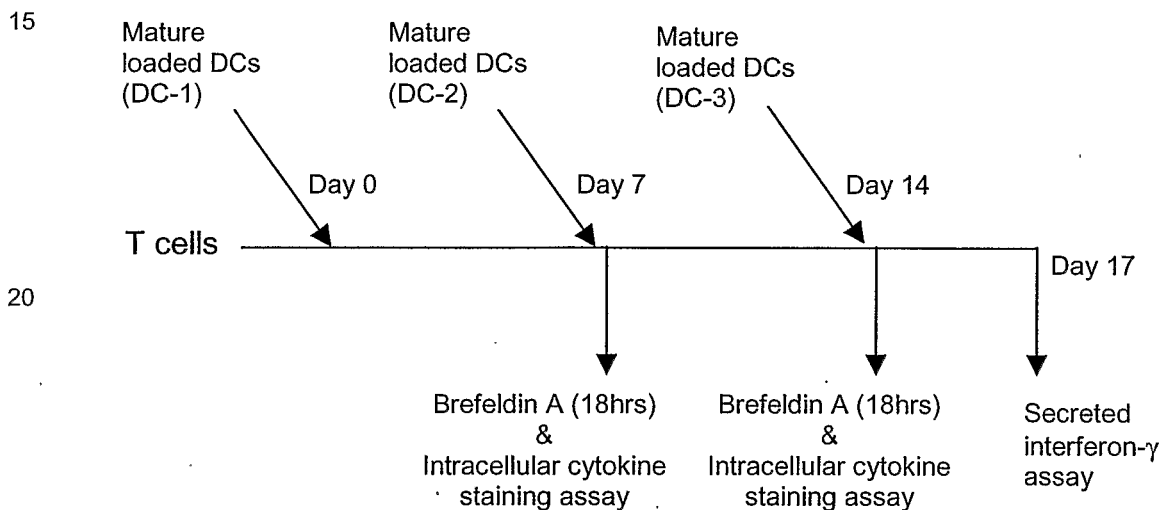
presence of TNF $\alpha$  and interferon- $\alpha$ . Autologous CD3<sup>+</sup> T cells were added to the mature dendritic cells. Following three rounds of exposure to the mature dendritic cells, T cell stimulation was quantitated by measuring the production of intracellular interferon- $\gamma$ , using flow cytometry. The levels of intracellular interferon- $\gamma$  produced in T cells in the presence of conjugate were substantially higher than in the presence of the sAg or the Fc fragment alone (Table 8).

**TABLE 8: T cell response to HbsAg-Fc DMS conjugate**

|                                                     | % IFN- $\gamma$ positive T cells |
|-----------------------------------------------------|----------------------------------|
| No antigen                                          | 0.19                             |
| Mouse Fc (2.5 $\mu$ g/mL)                           | 0.46                             |
| HBsAg (2.5 $\mu$ g/mL)                              | 0.04                             |
| HBsAg (2.5 $\mu$ g/mL) + mAb(2C12) (2.5 $\mu$ g/mL) | 0.12                             |
| HBsAg-Fc DMS conjugate (5.0 $\mu$ g/mL)             | 0.74                             |

#### H. Example 8: Antigen Presentation Assays

Antigen presentation assays were performed using human PBMC-derived dendritic cells according to established protocols (Berlyn, et al., *supra* (2001)). A protocol summary for the T cell stimulation assay is presented in schematic form.



#### 1. Preparation of Mature, Loaded Dendritic Cells

Monocytes were generated from leukapheresis samples from healthy donors and were depleted of lymphocytes and granulocytes by incubation with anti-CD2, CD7, CD16, CD19, and CD56 antibodies. This was followed by incubation with

magnetic bead conjugated anti-mouse IgG and separation on a magnet (Dyna). Negatively selected cells were greater than 95% pure monocytes as characterized by flow cytometry using a broad CD marker panel (CD14<sup>+</sup>, CD11c<sup>+</sup>, CD19<sup>-</sup>, CD3<sup>-</sup>, CD4<sup>-</sup>, CD64<sup>+</sup>, CD32<sup>+</sup>, CD86<sup>+</sup>, CD16<sup>-</sup>). Next, monocytes were incubated with IL-4 and GM-CSF (R&D Systems) for 4 days in AIM V plus 2.5% matched human serum to generate immature dendritic cells. Again, an aliquot of the cells was stained with the broad CD marker panel to ensure purity and identity of the cells. The cells then were loaded with HBV S1/S2-TBD (5.0 µg/ml), HBV S1/S2 (2.5 µg/ml), or TBD (2.5 µg/ml) for 2-4 hours at 37°C, and matured with interferon-α and TNF-α for 3 days. Dendritic cells were checked again using flow cytometry for an array of CD markers to ensure that cells had undergone proper maturation. The resulting mature, loaded dendritic cells were used for the T cell stimulation assay.

## 2. T cell Stimulation Assay: Cytokine Analysis

T cells were generated from the same sample of PMBCs as the dendritic cells by means of negative selection using a magnetic T cell isolation kit (Dyna) according to the manufacturer's directions. Mature, loaded dendritic cells (DC-1) were washed thoroughly and added to the T cells (Day 0). The T cells and dendritic cells were incubated for 7 days. On Day 7, the T cells were re-stimulated with mature, loaded dendritic cells (DC-2). An aliquot of the cells was taken 2 hours later. The aliquot of cells was incubated with Brefeldin A (GolgiPlug™, R&D Systems) for 18 hours and then assayed for intracellular cytokine staining as described below.

The remaining cells were incubated for another 7 days. On Day 14, the remaining cells were stimulated with a third batch of mature, loaded dendritic cells (DC-3). An aliquot of the cells was taken 2 hours later. The aliquot of cells was incubated with Brefeldin A (GolgiPlug™, R&D Systems) for 18 hours and then assayed for intracellular cytokine staining as described below.

For intracellular cytokine staining, cells were stained with anti-CD3-FITC and anti-CD8-Cy-Chrome for 30 minutes, washed, fixed, permeabilized, and then stained with anti-interferon-γ-PE for 30 minutes on ice. The cells were washed and analyzed by flow cytometry (FACScan, BD Biosciences). The results are shown in Table 9.

**TABLE 9: CD3<sup>+</sup>/IFN-γ<sup>+</sup> T cells**

|  |        |
|--|--------|
|  | Day 21 |
|--|--------|

|               |                 |
|---------------|-----------------|
| HBV S1/S2-TBD | $6.2 \pm 4.6$   |
| HBV S1/S2     | $1.9 \pm 1.7$   |
| TBD           | $1.6 \pm 0.9$   |
| No antigen    | $0.58 \pm 0.21$ |

After removal of the aliquot at Day 14, the remaining T cells were incubated for an additional three days and the supernatant then was used for measuring the level of secreted interferon- $\gamma$  by ELISA (Opt E1A ELISA kit, BD Biosciences). T cell stimulation was evaluated by measuring intracellular and secreted interferon- $\gamma$  levels. The results are presented in Table 10. The chimeric antigen S1/S2-TBD induced the production of higher interferon- $\gamma$  levels compared to either the immune response domain or the TBD domain of the molecule when tested alone, at equivalent concentrations. It should be pointed out that 5  $\mu$ g dose of S1/S2-TBD contains roughly 2.5  $\mu$ g each of the components.

**TABLE 10: Intracellular and Secreted Interferon- $\gamma$  Levels**

|               | % IFN- $\gamma$ positive T cells | Secreted IFN- $\gamma$ (pg/ml) |
|---------------|----------------------------------|--------------------------------|
| HBV S1/S2-TBD | 3.5                              | 60                             |
| HBV S1/S2     | 2.1                              | 18.9                           |
| TBD           | 2.5                              | 11.9                           |
| No antigen    | 0.77                             | 4.4                            |
| T cells alone | 0.21                             | 1.6                            |

Various concentrations of S1/S2-TBD were tested for the T cell response. The effect of S1/S2-TBD was greater than the tetanus toxoid treatment at similar concentrations. At concentrations lower than 5  $\mu$ g/mL, the chimeric antigen elicited a concentration dependent increase in the production and secretion of interferon- $\gamma$ . Interferon- $\gamma$  production and secretion by CD3<sup>+</sup> T cells increased in a concentration dependent manner following S1/S2-TBD antigen presentation by dendritic cells, as shown in Table 11. The positive response at low concentrations would be beneficial with respect to the dose necessary for vaccination and the cost of manufacturing of a vaccine.

**TABLE 11: Concentration Dependence of Response to Chimeric Antigen**

|                                 | % IFN- $\gamma$ positive T cells | Secreted IFN- $\gamma$ (pg/ml) |
|---------------------------------|----------------------------------|--------------------------------|
| HBV S1/S2-TBD (1.25 $\mu$ g/ml) | 1.5                              | 18                             |
| HBV S1/S2-TBD (2.5 $\mu$ g/ml)  | 4.3                              | 40                             |
| HBV S1/S2-TBD (5 $\mu$ g/ml)    | 3.5                              | 60                             |

|                          |      |     |
|--------------------------|------|-----|
| HBV S1/S2-TBD (10 µg/ml) | 4.3  | 20  |
| Tetanus toxoid           | 3.3  | 33  |
| No antigen               | 0.77 | 4.4 |
| T cells alone            | 0.21 | 1.6 |

## I. Example 9: Binding and Uptake of Chimeric Antigens

### 1. Preparation of Mature, Loaded Dendritic Cells

Peripheral blood mononuclear cells (PBMC) were obtained from  
 5 Ficoll/Histopaque (Sigma) treatment of a leukapheresis cell preparation (Berlyn, et al., *supra* (2001)). Monocytes were separated from the PBMC population by negative selection using a monocyte isolation kit (Dyna) following the manufacturer's directions. The monocytes were greater than 95% pure as assessed by antibody analysis and flow cytometry (CD14<sup>+</sup>, CD11c<sup>+</sup>, CD19<sup>-</sup>, CD3<sup>-</sup>, CD4<sup>-</sup>, CD64<sup>+</sup>, CD32<sup>+</sup>,  
 10 CD86<sup>+</sup>, CD16<sup>-</sup>). Monocytes were washed twice with AIM-V (Invitrogen) media containing L-glutamine, streptomycin sulfate (50 µg/mL) and gentamicin sulfate (10 µg/mL) with 1% donor matched sera (isolated as described in Berlyn, et al., *supra* (2001)). Next, the monocytes were cultured in AIM-V media containing 2.5% donor matched sera and the cytokines GM-CSF and IL-4 to differentiate the cells toward the  
 15 dendritic cell (DC) lineage. The cells were incubated in 12-well tissue culture plates at 37° C under a 7% CO<sub>2</sub> atmosphere.

The monocyte-derived dendritic cells were harvested on days 1 through 4. The cells were subsequently washed once with AIM-V media with 0.1% BSA (Sigma), and twice with Dulbecco's phosphate buffered saline (Invitrogen) with 0.1% (w/v)  
 20 BSA (PBSB). The monocyte-derived dendritic cells were used in 4° C labeling or binding assays or in 37° C binding/uptake assays.

### 2. Binding of Chimeric Antigens to Maturing Dendritic Cells

The extent of binding of S1/S2-TBD relative to murine IgG1 and IgG2a to maturing dendritic cells was compared. Dendritic cells were isolated at various days  
 25 of *ex vivo* culture (from day 0 to day 4) and treated with S1/S2-TBD (10 µg/mL) or with murine IgG1 (2C12, the parent mAb from which TBD was produced) or IgG2a (G155-178, 90 µg/mL) for 1 hour at 4°C. The cells were treated with F(ab')<sub>2</sub> goat anti-mouse Alexa-488 (10 µg/mL) in PBSB for 20 minutes. The cells were washed twice with PBSB and resuspended in PBSB with 2% paraformaldehyde (PF) and

acquired by a Becton Dickinson (BD) FACScan fitted with CellQuest acquisition and analysis software (BD). A gate was made on the viable cell population as determined by the FSC and SSC scatter profile and  $\geq 10,000$  events were acquired. To determine the percentage of positive cells, a gate was set based on negative control treated cells (isotype control labeled or cells labeled with  $F(ab')_2$  goat anti-mouse Alexa-488 alone). The percent of specific positive cells was calculated as:

$$\frac{\% \text{positive cells test sample} - \% \text{positive cells control}}{100 - \% \text{positive cells of control}} \times 100$$

The relative mean fluorescent intensity (MFI) was determined as the MFI of the test sample minus the MFI of the control sample.

The binding of S1/S2-TBD relative to IgG1 and IgG2a on DC after 1 to 4 days of culture is shown in Table 12.

**TABLE 12: Binding of Chimeric Antigen or Antibody to Maturing Dendritic Cells**

|               | % Specific positive dendritic cells |       |       |       |
|---------------|-------------------------------------|-------|-------|-------|
|               | Day 1                               | Day 2 | Day 3 | Day 4 |
| HBV S1/S2-TBD | 91.9                                | 98.5  | 88.3  | 97.9  |
| IgG1          | 21.2                                | 19.5  | 29.3  | 49.1  |
| IgG2a         | 28.0                                | 17.4  | 14.3  | 13.5  |

S1/S2-TBD binding was clearly much greater than the binding of either IgG1 or IgG2a with more S1/S2-TBD binding evident on day 1 than on day 4. These experiments demonstrated that S1/S2-TBD was bound with high efficiency to the maturing dendritic cells.

### 3. Uptake of Chimeric Antigens to Maturing Dendritic Cells

To determine the extent of uptake of chimeric antigens (*e.g.* HBV S1/S2-TBD) compared with IgG1 and IgG2a, cells were incubated with various concentrations of the antigen, IgG1 (2C12, the parent mAb from which TBD was produced) or IgG2a (G155-178) for 1 hour at 37° C in AIM V media with 0.1% BSA. Cells were washed twice in PBSB and fixed with PBS with 2% PF overnight at 4°C. Subsequently, the cells were washed twice in PBSB and permeabilized with PBS containing 0.1% (w/v) saponin (Sigma) for 40 minutes at 20°C.

The cells were washed twice with PBSB and incubated with F(ab')<sub>2</sub> goat anti-mouse Alexa-488 (10 µg/mL) in PBSB with 0.1% (w/v) saponin for 20 minutes at 4°C. After washing twice in PBSB, the cells were resuspended in PBSB. A variant of this assay involved treating the cells as above with chimeric antigen, IgG1, or IgG2a for 10 minutes followed by the addition of F(ab')<sub>2</sub> goat anti-mouse Alexa-488 (10 µg/mL) for 50 minutes. Subsequently the cells were washed and resuspended in PBS with 2% PF.

Cells were acquired by a Becton Dickinson (BD) FACScan fitted with Cellquest acquisition and analysis software (BD). A gate was made on the viable cell population as determined by the FSC and SSC scatter profile and ≥10,000 events were acquired. To determine the percentage of positive cells, a gate was set based on negative control treated cells (isotype control labeled or cells labeled with F(ab')<sub>2</sub> goat anti-mouse Alexa-488 alone). The percent of specific positive cells was calculated as:

$$\frac{\% \text{positive cells test sample} - \% \text{positive cells control}}{100 - \% \text{positive cells of control}} \times 100$$

The relative mean fluorescent intensity (MFI) was determined as the MFI of the test sample minus the MFI of the control sample.

The uptake of S1/S2-TBD in comparison to murine IgG1 and IgG2a was estimated as a function of concentration on day 4 of dendritic cells maturation. The uptake was quantified at 37° C for 1 hour and the results are shown in FIG. 4. There was a linear increase in the uptake of S1/S2-TBD with concentration. IgG1 was taken up at a much lower level and there was very little uptake of IgG2a. Therefore, the chimeric antigen S1/S2-TBD is taken up by the dendritic cells more efficiently than immunoglobulins.

#### **J. Example 10: Expression of Fc-γ Receptors and CD206 on Maturing DC**

There are several receptors on the antigen presenting cells that bind and take up antigens. The abundance of these receptors on maturing dendritic cells was evaluated using fluorescent labeled receptor-specific antibodies. FACS analysis was used to estimate percentage of specific receptor positive cells in the total population of dendritic cells. The degree of receptor expression was assessed by determination of the relative mean fluorescent intensity and as a function of relative fluorescent intensity (Table 13).

**TABLE 13: Expression of Antigen Binding Receptors on Maturing Dendritic Cells**

|       | % specific positive cells |       |       |       |       | Relative MFI |       |        |        |        |
|-------|---------------------------|-------|-------|-------|-------|--------------|-------|--------|--------|--------|
|       | Day 0                     | Day 1 | Day 2 | Day 3 | Day 4 | Day 0        | Day 1 | Day 2  | Day 3  | Day 4  |
| CD16  | 20.8                      | 26.3  | 6.2   | 0.8   | 5.9   | 4.3          | 10.8  | 2.7    | 0.0    | 1.6    |
| CD32  | 99.3                      | 97.4  | 78.9  | 41.8  | 37.8  | 163.4        | 187.1 | 70.5   | 18.0   | 14.0   |
| CD64  | 84.0                      | 71.9  | 18.2  | 9.6   | 5.3   | 28.0         | 12.8  | 3.7    | 2.7    | 1.0    |
| CD206 | 45.8                      | 82.5  | 98.3  | 99.1  | 99.3  | 8.5          | 373.1 | 1180.6 | 1317.3 | 1680.4 |

5           The expression of CD64 (Fcγ receptor I) decreased with time in culture and at day 4 was almost negligible. In contrast, CD32 (Fcγ receptor II), and to a lesser extent CD16(Fcγ receptor III), continued to be expressed after 4 days of DC culture. On day 0 of culture, there was essentially no CD206 (mannose macrophage receptor) expression. But expression was induced upon culture with IL-4 and GM-CSF, and by  
10   day 4 CD206 was expressed at very high levels. Thus at day 4, when antigen was loaded in the antigen presentation assays, the dendritic cells possessed at least two potential receptors for the binding of chimeric antigens: CD32 and CD206. In addition, they had the full complement of the co-stimulatory molecules (data not shown). The expression of HLA-DR (Class II) and HLA-ABC (Class I) also increased  
15   with time in culture. Co-stimulatory molecules CD86 (B7.2) and CD80 (B7.1) were expressed throughout the period of the assay. These results indicate that the monocyte-derived dendritic cells were differentiating towards mature dendritic cells and were capable of antigen processing and presentation to T cells.

#### **K. Example 11: Correlation of CD32/CD206 Expression and S1/S2-TBD**

##### **20   Binding to Maturing DCs**

There is a direct correlation between the expression of CD32/CD206 receptors and S1/S2-TBD binding to maturing dendritic cells. Since it was known that murine IgG1 binds to human CD32, it was expected that S1/S2-TBD, which contains the murine Fc component of IgG1, would also bind CD32. Furthermore, S1/S2-TBD by  
25   virtue of its high mannose glycosylation, would also be expected to bind to dendritic cells through the CD206 receptor.

The dot plots in FIG. 5 show S1/S2-TBD binding (10 µg/mL) and CD32 expression as well as S1/S2-TBD binding and CD206 expression. There was a direct

correlation between the extent of S1/S2-TBD binding and the degree of CD32 expression, which was relatively heterogeneous, *i.e.*, there was a broad degree of expression. These results demonstrate that S1/S2-TBD binds to CD32, and that the greater the expression of CD32, the greater was the degree of binding of the chimeric antigen S1/S2-TBD. The dot plot of S1/S2-TBD binding and CD206 expression shows that the vast majority of cells expressing CD206 also bound S1/S2-TBD. A small percentage of the cell population was CD206 negative and was consequently negative for S1/S2-TBD binding. Therefore both CD32 and CD206 receptors correlate with the binding of S1/S2-TBD.

**L. Example 12: Binding and Uptake of S1/S2-TBD is Primarily Via CD32 with CD206 Involved to a Lesser Extent**

The uptake of S1/S2-TBD in comparison to murine IgG1 and IgG2a was estimated as a function of concentration on day 4 of DC maturation. The uptake was quantified at 37° C for 1 hour in the presence media, mannan (2 mg/ml, Sigma), and/or mouse Fcγ (2 mg/ml, Jackson ImmunoResearch Laboratories). Mannan is a competitive inhibitor of CD206 binding and therefore of uptake of antigens via CD206 on dendritic cells. Fcγ is a competitive inhibitor of CD32 binding and therefore CD32-mediated antigen uptake. The results are shown in Table 14.

**TABLE 14: Inhibition of Chimeric Antigen Binding by Fc or Mannan**

|                         | Relative MFI |           |              |       |
|-------------------------|--------------|-----------|--------------|-------|
|                         | Mannan       | Mouse Fcγ | Mannan & Fcγ | Media |
| 0.5 µg/ml HBV S1/S2-TBD | 7.6          | 0.5       | 0.6          | 3.0   |
| 2.5 µg/ml HBV S1/S2-TBD | 21.5         | 2.0       | 3.3          | 22.6  |
| 6 µg/ml HBV S1/S2-TBD   | 41.6         | 5.7       | 5.0          | 49.2  |

There was a progressive increase in the binding of the chimeric antigen with its concentration. Incubation of the cells with a high concentration of mouse Fcγ fragment abolished this binding, whereas mannan, an inhibitor of CD206 receptor binding, had only a marginal effect. Therefore, CD32 may be the primary receptor involved in the binding and uptake of the chimeric antigen.

### M. Example 13: Glycosylation of HBV S1/S2 Antigen Imparts Immunogenicity

The insect cell pathway of protein glycosylation is different from that of mammalian cells in that proteins synthesized in insect cells undergo glycosylation that results in high mannose content and a lack of terminal sialic acid residues in the secreted protein (Altman, et al., *Glycoconj* 16:109-123 (1999)). HBV S1/S2, the antigen component of the chimeric antigen was expressed in both *E. coli* (no glycosylation) and in High Five™ insect cells (mannose glycosylation).

#### 1. Effect of glycosylation on binding of antigen

These antigens were compared for their binding to dendritic cells, as described in Example 9. Maturing dendritic cells were loaded with 10 µg/ml of HBV S1/S2 expressed in insect cells or in *E. coli*. Glycosylated protein showed better binding by dendritic cells (Table 15).

TABLE 15: Effect of Glycosylation on Binding of HBV S1/S2

|                | % Specific positive cells | Relative MFI |
|----------------|---------------------------|--------------|
| Insect cells   | 69.9                      | 40.3         |
| <i>E. coli</i> | 12.2                      | 3.9          |

#### 2. Effect of glycosylation on eliciting immune response

Glycosylation of HBV S1/S2 elicits increased immunogenicity and T Cell responses. HBV S1/S2, expressed in both *E. coli* and High Five™ insect cells, were compared for T cell responses when presented by dendritic cells. Both intracellular and secreted interferon-γ levels were measured, as described in Example 8 (using 2.5 µg/ml HBV S1/S2 protein), and the results are presented in Table 16.

Table 16: Effect of Glycosylation on Interferon-γ Levels

|                          | Intracellular IFNγ<br>(% IFNγ positive T cells) | Secreted IFNγ<br>(pg/ml) |
|--------------------------|-------------------------------------------------|--------------------------|
| Baculovirus HBV S1/S2    | 2.1                                             | 18.9                     |
| <i>E. coli</i> HBV S1/S2 | 0.83                                            | 4.3                      |
| No antigen               | 0.77                                            | 4.4                      |
| T cells alone            | 0.21                                            | 1.6                      |

HBV S1/S2 expressed in insect cells generated a higher level of both intracellular and secreted interferon, as compared to the unglycosylated protein expressed in *E. coli*.

5 All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood  
10 that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention, which are obvious to those skilled in the art, are intended to be within the scope of the following claims.

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To (Name of Depositor): Irwin Griffith, PhD ViRexx Medical Corp.

Address: 1123 Dentistry/Pharmacy Bldg, University of Alberta, Edmonton, AB T6G  
2N8**Identification of Deposit**Reference assigned by depositor: ~~pFastBacHTa~~ HCV E2-TBD

Accession Number assigned by this IDA: 080504-01

The deposit identified above was accompanied by:

☐ a scientific description (specify):☐ a proposed taxonomic designation (specify):

Signature of person(s) authorized to represent IDAC:

Date: August 05, 2004

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To (Name of Depositor): Irwin Griffith, PhD ViRexx Medical Corp.

Address: 1123 Dentistry/Pharmacy Bldg, University of Alberta, Edmonton, AB T6G  
2N8**Identification of Deposit**

Reference assigned by depositor: pFastBacHTa HCV NS5A-TBD

Accession Number assigned by this IDA: 080504-02

The deposit identified above was accompanied by:

☐ a scientific description (specify):☐ a proposed taxonomic designation (specify):

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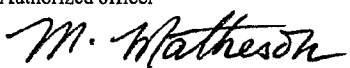
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Address: 1123 Dentistry/Pharmacy Bldg, University of Alberta, Edmonton, AB T6G  
2N8**Identification of Deposit**Reference assigned by depositor: pFastBa~~ac~~HTa HBV S1/S2-TBD

Accession Number assigned by this IDA: 080504-03

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Reference assigned by depositor: pFastBacHTa-HBV core-TBD

Accession Number assigned by this IDA: 080504-04

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Address: 1123 Dentistry/Pharmacy Bldg, University of Alberta, Edmonton, AB T6G  
2N8**Identification of Deposit**

Reference assigned by depositor: pFastBacHTa HCV core(1-177) TBD

Accession Number assigned by this IDA: 080504-05

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☐ a scientific description (specify):☐ a proposed taxonomic designation (specify):

Signature of person(s) authorized to represent IDAC:

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**WHAT IS CLAIMED IS:**

1. A chimeric antigen for eliciting an immune response comprising an immune response domain and a target binding domain, wherein the target binding domain comprises an antibody fragment.
2. The chimeric antigen of claim 1, wherein the antibody fragment is a xenotypic antibody fragment.
3. The chimeric antigen of claim 1, wherein the antibody fragment is not a xenotypic antibody fragment.
4. The chimeric antigen of claim 1, wherein the chimeric antigen comprises more than one immune response domain.
5. The chimeric antigen of claim 1, wherein the immune response domain comprises at least one antigenic portion of a protein selected from the group consisting a HBV protein, a DHBV protein and a HCV protein.
6. The chimeric antigen of claim 5, wherein the immune response domain comprises at least one antigenic portion of a HBV protein selected from the group consisting of HBV S1/S2, HBV S1/S2/S, HBV Core, HBV Core ctm and HBV polymerase.
7. The chimeric antigen of claim 5, wherein the immune response domain comprises at least one antigenic portion of a DHBV protein selected from the group consisting of DHBV PreS/S, DHBV PreS, DHBV Core and DHBV polymerase.
8. The chimeric antigen of claim 5, wherein the immune response domain comprises at least one antigenic portion of a HCV protein selected from the group consisting of HCV Core (1-191), HCV Core (1-177), HCV E1-E2, HCV E1, HCV E2, HCV NS3, HCV NS5A and HCV NS4A.
9. The chimeric antigen of any of claims 1-8, wherein the immune response domain further comprises a 6xHis tag fused to the protein.

10. The chimeric antigen of any of claims 1-9 wherein the immune response domain comprises one or more antigenic portions of a protein from HPV, HIV, HSV, an obligate intracellular parasite or a cancer antigen.
11. The chimeric antigen of any of claims 1-10, wherein the target binding domain is capable of binding to an antigen presenting cell.
12. The chimeric antigen of any of claims 1-11, wherein the antibody fragment comprises an immunoglobulin heavy chain fragment.
13. The chimeric antigen of claim 12, wherein the immunoglobulin heavy chain fragment comprises a hinge region.
14. The chimeric antigen of claim 12, wherein the chimeric antigen further comprises amino acids, VDKKI (SEQ ID NO: 2).
15. The chimeric antigen of claim 12, wherein the immunoglobulin heavy chain fragment comprises C<sub>H</sub>2 and C<sub>H</sub>3 domains.
16. The chimeric antigen of any of claims 1-15, wherein the antibody fragment is a Fc fragment.
17. The chimeric antigen of any of claims 1-15, wherein the antibody fragment comprises a portion of a C<sub>H</sub>1 region and an antibody hinge region.
18. The chimeric antigen of any of claims 1-17, further comprising a linker for linking the immune response domain and the target binding domain.
19. The chimeric antigen of claim 18, wherein the linker is a covalent peptide linkage.
20. The chimeric antigen of claim 19, wherein the peptide linkage comprises the sequence SRPQGGGS or VRPQGGGS ( SEQ ID NO: 1).
21. The chimeric antigen of claim 18, wherein the linker is selected from the group consisting of leucine zippers and biotin/avidin.

22. The chimeric antigen of any of claims 1-21, wherein the chimeric antigen is mannose glycosylated.
23. The chimeric antigen of claim 22, wherein the immune response domain is mannose glycosylated.
24. The chimeric antigen of claim 22, wherein the target binding domain is mannose glycosylated.
25. The chimeric antigen of claim 22, wherein the chimeric antigen is pauci mannose glycosylated.
26. The chimeric antigen of claim 22, wherein the chimeric antigen is high mannose glycosylated.
27. The chimeric antigen of claim 1, wherein the target binding domain comprises amino acid sequences SRPQGGGS (SEQ ID NO: 1), VDKKI (SEQ ID NO: 2) and C<sub>H</sub>2 and C<sub>H</sub>3 domains of the murine immunoglobulin heavy chain constant region.
28. The chimeric antigen of any of claims 1-27, which elicits a multi-epitopic response.
29. The chimeric antigen of claim 1, which elicits an immune response to at least one epitope of the immune response domain.
30. The chimeric antigen of any of claims 1-29, which elicits a humoral immune response.
31. The chimeric antigen of any of claims 1-29, which elicits a cellular immune response.
32. The chimeric antigen of any of claims 1-29, which elicits a Th1 immune response, a Th2 immune response, a CTL response, or a combination thereof.
33. A method of enhancing antigen presentation in an antigen presenting cell comprising contacting the antigen presenting cell with a composition comprising a chimeric antigen of any of claims 1-32.

34. The method of claim 33, wherein the antigen presenting cell is a dendritic cell.
35. The method of claim 34, wherein the method enhances antigen presentation of more than one epitope.
36. The method of claim 34, wherein the method enhances antigen presentation of at least one epitope of the immune response domain.
37. A method of activating an antigen presenting cell comprising contacting the antigen presenting cell with a chimeric antigen of any of claims 1-32.
38. The method of claim 33 or 37, wherein the contacting takes place *ex vivo*.
39. The method of claim 33 or 37, wherein the contacting takes places *in vivo*.
40. The method of claim 39, wherein the contacting takes place in a human.
41. A method of eliciting an immune response comprising administering to a subject a composition comprising a chimeric antigen of any of claims 1-32.
42. A method of breaking tolerance comprising contacting an antigen presenting cell with a chimeric antigen of any of claims 1-32.
43. A method for treating an immune-treatable condition comprising administering, to a subject in need thereof, a chimeric antigen of any of claims 1-32.
44. The method according to any of claims 41-43, wherein administration of the immune response domain alone does not elicit an immune response.
45. The method according to any of claims 41-43, wherein administration of the chimeric antigen elicits a greater immune response than administration of the immune response domain alone.
46. The method of claim 45, wherein the immune response is measured by the amount of antigen-specific antibody present in the subject.
47. The method of claim 45, wherein the immune response is measured by the amount of interferon- $\gamma$  secreted by T cells in response to being exposed to antigen presenting cell loaded with the chimeric antigen or immune response domain alone.

48. The method of claim 45, wherein the immune response is measure by the amount of antigen-specific CD8<sup>+</sup> T cells elicited in response to being exposed to antigen presenting cell loaded with the chimeric antigen or immune response domain alone.
49. The method according to any of claims 41-43, wherein the subject has an infection or a cancer.
50. The method of claim 49, wherein the infection is a viral infection or a parasitic infection.
51. The method of claim 50, wherein the infection is a chronic viral infection.
52. The method of claim 51, wherein the chronic viral infection is a chronic hepatitis B viral infection or a chronic hepatitis C viral infection, a chronic human papilloma viral infection, a chronic human immunodeficiency viral infection, or a chronic herpes simplex viral infection .
53. The method of claim 52, wherein the infection is a hepatitis B viral infection and the immune response domain comprises at least one antigenic portion of a protein selected from the group consisting of HBV Core protein, a HBV S protein, a HBV S1 protein and a HBV S2 protein and combinations thereof.
54. The method of claim 52, wherein the infection is a hepatitis C viral infection and the immune response domain comprises at least one antigenic portion of a protein selected from the group consisting of a HCV core (1-191) protein, a HCV Core (1-177) protein, a HCV E1 protein, a HCV E2 protein, a HCV E1-E2 protein, a HCV NS3 protein, a HCV NS5A protein and combinations thereof.
55. A method of vaccinating a subject against a viral infection comprising administering to the subject, a chimeric antigen of any of claims 1-32.
56. The method of claim 55, wherein the subject is therapeutically vaccinated against an existing viral infection.
57. The method of claim 55, wherein the subject is prophylactically vaccinated against a viral infection.

58. The method of claim 55, wherein the subject develops an immune response to more than one epitope of the chimeric antigen.
59. The method of claim 58, wherein the subject develops an immune response to more than one epitope of the immune response domain.
60. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a chimeric antigen of any of claims 1-32.
61. The pharmaceutical composition of claim 60, wherein the pharmaceutical composition is formulated for parenteral administration.
62. The pharmaceutical composition of claim 60, wherein the pharmaceutical composition is formulated for transdermal, intradermal, intravenous, subcutaneous, intramuscular, nasal, pulmonary or oral administration.
63. An article of manufacture comprising a chimeric antigen according to any of claims 1-32 and instructions for administering the chimeric antigen to a subject in need thereof.
64. A method of producing a chimeric antigen comprising:
- (a) providing a microorganism or cell line, which comprises a polynucleotide that encodes a chimeric antigen according to any of claims 1-32; and
  - (b) culturing said microorganism or cell line under conditions whereby the chimeric antigen is expressed.
65. The method of claim 64, wherein the microorganism or cell line is a eukaryotic microorganism or cell line.
66. The method of claim 65, wherein the microorganism or cell line is a yeast, a plant cell line or an insect cell line.
67. The method of claim 64, wherein the chimeric antigen is post-translationally modified to comprise a mannose glycosylation.
68. A polynucleotide encoding a chimeric antigen, said polynucleotide comprising a first polynucleotide portion encoding an immune response domain and a second

polynucleotide portion encoding a target binding domain, wherein the target binding domain comprises an antibody fragment.

69. The polynucleotide of claim 68, wherein the immune response domain comprises at least one antigenic portion of a viral protein.

70. The polynucleotide of claim 69, wherein the viral protein is a HBV protein or a HCV protein.

71. The polynucleotide of claim 68, wherein the antibody fragment is a xenotypic fragment.

72. The polynucleotide of claim 68, wherein the antibody fragment is not a xenotypic fragment.

73. The polynucleotide of claim 68, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of nucleotides 1 to 1326 of SEQ ID NO: 26, nucleotides 1 to 2004 of SEQ ID NO: 28, nucleotides 1 to 1350 of SEQ ID NO: 30, nucleotides 1 to 1293 of SEQ ID NO: 32, nucleotides 1 to 1794 of SEQ ID NO: 34, nucleotides 1 to 1581 of SEQ ID NO: 36, nucleotides 1 to 1389 of SEQ ID NO: 38, nucleotides 1 to 1347 of SEQ ID NO: 40, nucleotides 1 to 2157 of SEQ ID NO: 42, nucleotides 1 to 1395 of SEQ ID NO: 44, nucleotides 1 to 1905 of SEQ ID NO: 46 and nucleotides 1 to 2484 of SEQ ID NO: 48.

74. The polynucleotide of claim 68, wherein the polynucleotide encodes a chimeric antigen that is at least 90% identical to an entire amino acid sequence selected from the group consisting of amino acids 1 to 442 of SEQ ID NO: 27, amino acids 1 to 668 of SEQ ID NO: 29, amino acids 1 to 450 of SEQ ID NO: 31, amino acids 1 to 431 of SEQ ID NO: 33, amino acids 1 to 598 of SEQ ID NO: 35, amino acids 1 to 527 of SEQ ID NO: 37, amino acids 1 to 463 of SEQ ID NO: 39, amino acids 1 to 449 of SEQ ID NO: 41, amino acids 1 to 719 of SEQ ID NO: 43, amino acids 1 to 465 of SEQ ID NO: 45, amino acids 1 to 635 of SEQ ID NO: 47 and amino acids 1 to 828 of SEQ ID NO: 49.

75. The polynucleotide of claim 68, wherein the polynucleotide selectively hybridizes under stringent conditions to a polynucleotide having a nucleotide

sequence selected from the group consisting of SEQ ID NO: 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 and 48

76. A microorganism or cell line comprising the polynucleotide of claim 68.

77. A vector comprising the polynucleotide of claim 68.