HEPATITIS B VIRUS CORE PROTEIN AND SURFACE ANTIGEN PROTEIN AND VACCINE COMPRISING THE SAME

FIELD OF THE INVENTION

[0001] The present invention relates to nucleic acid sequences encoding hepatitis B virus (HBV) core proteins, surface antigen proteins, fragments and combinations thereof, to hepatitis B virus (HBV) core proteins, surface antigen proteins, fragments and combinations thereof, to improved HBV vaccines, to improved methods for inducing immune responses against HBV, and to improved methods for prophylactically and/or therapeutically immunizing individuals against HBV.

BACKGROUND

[0002] Hepatitis B is a common infection prevalent across the globe that leads to the development of cirrhosis, liver failure, and hepatocellular carcinoma. A significant number of hepatitis cases go unreported due to the asymptomatic nature of the disease. Nevertheless, about 350 million chronic Hepatitis B cases are reported every year. Most of the hepatitis infected population is in underdeveloped or developing countries.

[0003] The virus is divided into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes present on its envelope proteins. There are at least eight genotypes (A-H) of HBV according to variation of the genomic sequences. The alternative genotypes of HBV have prevalent geographic distribution.

[0004] Table 1 shows the geographic distribution of HBV genotypes.

Table 1 Geographic Distribution of HBV

HBV genotype	*HBV genosubtype*	*HBsAg subtype*	*Frequency*	*Main geographical distribution*
A	A2	adw2	High	Europe, North America, Australia
	Al	ayw1, adw2	High	Africa
B	B1 B2, B3	adw2	High	Far East
	B4	ayw1	High	Far East
	B2	adw3	Low	Far East
C	C1, C2, C4	adr	High	Far East
	C3	adrq-	High	New Guinea., Pacific
	C1, C2	ayr	High	Far East
	C1, C3	adw2	Low	Far East

	C4	ayw3	Low	Far East, Pacific
D	D1, D3, D4	ayw2	High	West Asia, Eastern Europe, Mediterranean
	D2, D3	ayw3	High	Worldwide
	Not identified	adw3	Low	Eastern Europe, Spain
	D2	ayw4	Low	Eastern Europe, Spain, United States
E	-	ayw4	High	Africa
F	F1, F2	adw4q-	High	Latin America, Alaska, Pacific
	F1, F2	ayw4	Low	Latin America
G	-	adw2	Low	Europe, North America
H	-	ayw4	Low	Central America

J. Med. Virol, DOI 10.1002jmv

[0005] The HBV genome is a circular DNA molecule that is primarily double stranded but which has a single stranded region arising from one strand being longer than the other. The double stranded region arises from the hybridization of a shorter strand of about 3020 nucleotides to a longer strand of about 3320 nucleotides. The single stranded region on non-hybridized nucleotides of the longer strand is associated with the HBV DNA polymerase. The HBV genomic DNA and HBV DNA polymerase are both contained within a nucleocapsid formed by multiple HBV core protein (HBcAg) molecules. The HBV core protein is enveloped by HBV surface protein or antigen (HBsAgs) and lipid molecules.

[0006] The HBV genome contains four open reading frames (ORFs) that encode seven different proteins: 1) an ORF that encodes the HBV DNA polymerase, 2) an ORF that has two start codons, wherein the sequence linked to the second start codon encodes the core protein and the sequence that includes the additional upstream start codon encodes a sequence referred to as pre-C; 3) an ORF that has three start codons, wherein one encodes the surface protein (S protein; gp27), one includes an upstream start codon which encodes a sequence referred to as pre-S2 (gp36) and another which includes a start codon further upstream which encodes a sequence referred to as pre-S1 (gp42); and 4) an ORF that encodes HBxAg, a protein whose function is less understood (Figure 1). These include 3 surface proteins or antigens (HBsAg): preS1, preS2 and the major S.

[0007] Features of the HBsAgs are illustrated in Figure 2. Epitopes of the HBsAgs involved in the expression of subtype specificities are located in a region that includes the two external loops of the HBV surface antigen molecules (i.e., amino acids 110-180 of S protein) and are what make the HBV strains antigenically diverse. The same region contains

an unknown number of epitopes that define the "a" determinant of HBsAg, which is common to all of the HBV wild-type strains known.

[0008] Prophylactic vaccines and therapies for HBV infection involve injection of subviral particles purified from plasma of chronic carriers, or subviral particles produced as recombinant proteins in stably transfected eukaryotic cell lines. The subviral particles are viral proteins and such vaccines are often referred to as subunit vaccines. The HBV proteins are administered to an individual and become targets for the individual's immune system. In uninfected individuals, an immune response against the subunit vaccine protects the uninfected individual from HBV infection. In infected individuals, the immune response induced by the vaccine can have therapeutic effects.

[0009] Chisari F.V., Am J Pathol., 2000. 156:1117-1132 and Pumpeus P. et al. Intervirology 2001. 44:98-114 disclose HBV genomic organization. Deny P. and F. Zoulim, Pathologie Biologie 2010, Aug, 58(4):245 53 discuss hepatitis B virus diagnosis and treatment. Michel M.L. and P. Tiollais, Pathologie Biologie 2010, Aug, 58(4):288 95 discuss hepatitis B vaccines and their protective efficacy and therapeutic potential. PCT publication WO2004026899 discloses the use of immunogen containing polypeptide sequence with HBV amino acid sequences. PCT published application WO2008093976 discloses HBV coding sequences, proteins and vaccines including a vaccine comprising a recombinant full length HBV surface antigen and HBV core antigen. The entire HBV surface antigen consists of three types of surface protein (L protein, M protein and S protein). PCT published application WO2009130588 discloses HBV coding sequences, proteins and vaccines including a nucleic acid encoding a hepatitis B virus core antigen that is codon optimized for expression in humans. PCT publication WO2010127115 discloses delivery of HBV sequences using recombinant vectors.

[0010] The available HBV vaccines have exhibited some efficacy, but are costly to produce. In addition, plasma-derived subunit vaccines also have concerns about safety. Several vaccine approaches have been explored including those based on recombinant live vectors, synthetic peptides, and DNA vaccines that comprise codon optimized coding sequences of HBV proteins. These other approaches have thus far had varying limited efficacy. Additionally, due to genomic differences, some HBV vaccines have exhibited positive efficacy in some geographic areas and limited efficacy in other areas.

[0011] Currently available HBsAg-based vaccines derived from yeast transfected with DNA encoding S protein (e.g., ENGERIX-B available from SmithKline Biologicals located

in Belgium and RECOMBIVAX/HB-VAX II available from Merck & Co. located in the U.S.A.) do not elicit a response in about 5% to 10% of individuals (C. Belloni, Immunogenicity of hepatitis B vaccine in term and preterm infants. Acta Paediatrica, 1998. 87: p. 336-338). Additionally, the rate of non-response increases to 30% in older individuals and immunity against HBV can decrease several years after vaccination. Multiple doses are also used to attain complete protection. Safety is of concern with the HBsAg-based vaccine ENGERIX-B as ENGERIX-B triples the risk of central nervous system (CNS) inflammatory demyelination.

[0012] Other HBsAg-based vaccines derived from mammalian cells utilize pre-S1 and pre-S2 in addition to S protein. The pre-S1 and –S2 antigens express highly immunogenic T and B cell epitopes and one such vaccine elicits an immune response in about 80% of non- or low-responding individuals (Rendi-Wagner, P. et al., Comparative immunogenicity of PreS/S hepatitis B vaccine in non- and low responders to conventional vaccine. Vaccine, 2006. 24: p. 2781-9.).

[0013] The direct administration of nucleic acid sequences to vaccinate against animal and human diseases has been studied and much effort has focused on effective and efficient means of nucleic acid delivery in order to yield necessary expression of the desired antigens, resulting immunogenic response and ultimately the success of this technique.

[0014] DNA vaccines allow for endogenous antigen synthesis, which induces CD8+ histocompatible complex, class I-restricted cytotoxic T lymphocytes that are rarely obtained with subunit vaccines. In addition, the antigen synthesis that occurs over a sustained period can help overcome low responsiveness and eliminate or reduce the requirement for booster injections. Further, DNA vaccines appear to be very stable and simple to produce.

[0015] DNA vaccines are safe, stable, easily produced, and well tolerated in humans with preclinical trials indicating little evidence of plasmid integration [Martin, T., et al., Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. Hum Gene Ther, 1999. 10(5): p. 759-68; Nichols, W.W., et al., Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci, 1995. 772: p. 30-9]. In addition, DNA vaccines are well suited for repeated administration due to the fact that efficacy of the vaccine is not influenced by pre-existing antibody titers to the vector [Chattergoon, M., J. Boyer, and D.B. Weiner, Genetic immunization: a new era in vaccines and immune therapeutics. FASEB J, 1997. 11(10): p. 753-63]. However, one major obstacle for the clinical adoption of DNA vaccines has been a decrease in the platform's immunogenicity

when moving to larger animals [Liu, M.A. and J.B. Ulmer, Human clinical trials of plasmid DNA vaccines. Adv Genet, 2005. 55: p. 25-40].

[0016] Recent technological advances in the engineering of DNA vaccine immunogen have improved expression and immunogenicity of DNA vaccines, such has codon optimization, RNA optimization and the addition of immunoglobulin leader sequences [Andre, S., et al., Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. J Virol, 1998. 72(2): p. 1497-503; Deml, L., et al., Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. J Virol, 2001. 75(22): p. 10991-1001; Laddy, D.J., et al., Immunogenicity of novel consensus-based DNA vaccines against avian influenza. Vaccine, 2007. 25(16): p. 2984-9; Frelin, L., et al., Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4A gene. Gene Ther, 2004. 11(6): p. 522-33], as well as, recently developed technology in plasmid delivery systems such as electroporation [Hirao, L.A., et al., Intradermal/subcutaneous immunization by electroporation improves plasmid vaccine delivery and potency in pigs and rhesus macaques. Vaccine, 2008. 26(3): p. 440-8; Luckay, A., et al., Effect of plasmid DNA vaccine design and *in vivo* electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J Virol, 2007. 81(10): p. 5257-69; Ahlen, G., et al., *In vivo* electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ T cells. J Immunol, 2007. 179(7): p. 4741-53]. The *in vivo* electroporation technique has been used in human clinical trials to deliver anti-cancer drugs, such as bleomycin, and in many preclinical studies on a large number of animal species. In addition, studies have suggested that the use of consensus immunogens can be able to increase the breadth of the cellular immune response as compared to native antigens alone [Yan, J., et al., Enhanced cellular immune responses elicited by an engineered HIV-1 subtype B consensus-based envelope DNA vaccine. Mol Ther, 2007. 15(2): p. 411-21; Rolland, M., et al., Reconstruction and function of ancestral center-of-tree human immunodeficiency virus type 1 proteins. J Virol, 2007. 81(16): p. 8507-14].

[0017] Antibodies and cytotoxic T cells (CTLs) to different HBV antigens may be involved in reducing viral load and clearing HBV infected cells from the liver. A major challenge for current vaccine candidates has been the induction of both humoral and cellular immunity to multiple HBV antigen targets and against the major genotypes of HBV. There

remains a need for nucleic acid constructs that encode HBV protein and for compositions useful to induce immune responses against HBV. There remains a need for effective vaccines against HBV that are economical and effective. There remains a need for effective vaccines that increase neutralizing antibody levels and elicit a T-cell component. There remains a need for effective vaccines against HBV, including those that are effective against HBV strains having a broad range of genotypes, and preferably, a universal vaccine that would be globally effective.

SUMMARY OF INVENTION

[0018] The present invention is directed to a vaccine comprising: (a) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6; (b) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO:10, SEQ ID NO:14, a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:10, and a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:14; and (c) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO: 12, SEQ ID NO:16, a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:12, and a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:16. The nucleic acid molecules can comprise one or more nucleotide sequences selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:15.

[0019] The present invention may also be directed to a vaccine comprising (a) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6; (b) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO:10, SEQ ID NO:14, a protein that is 95% identical over the entire length of the amino acid sequence of the full length amino acid sequence of SEQ ID NO:10, and a protein that is 95% identical over the entire length of the amino acid sequence of the full length amino acid sequence of SEQ ID NO:14; and (c) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO: 12, SEQ ID NO:16, a protein that is 95% identical over the entire length of the amino acid sequence of the full length amino acid sequence of SEQ ID NO:12, and a protein that is 95% identical over the entire length of the amino acid sequence of the full length amino acid sequence of SEQ ID NO:16.

[0020] The vaccine can be a plasmid comprising the nucleic acids described above. The nucleic acid molecules can be incorporated into viral particles. The vaccine can further comprise an adjuvant molecule. The adjuvant can be IL-12, IL-15, IL-28, CTACK, TECK, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-18, IL-21, IL-31, IL-33 or a combination thereof; and in some embodiments, IL-12, IL-15, IL-28, or RANTES.

[0021] The present invention is also directed to a method of inducing an immune response against an HBV antigen comprising administering a vaccine of the present invention to a subject.

[0022] The present invention is also directed to a method of protecting a subject from HBV infection comprising administering a vaccine of the present invention to the subject.

[0023] The present invention is further directed to a method of protecting a subject who has been diagnosed with HBV infection comprising administering a vaccine of the present invention to the subject.

[0024] The present invention further includes vaccines useful for inducing an immune response against HBV. The development of an HBV immune therapeutic vaccine with broad effectiveness against a multitude of genotypes can be provided using a therapeutic DNA vaccine for HBV infection based on targeting the universally conserved HBV-core specific antigens. The utilization of consensus HBV immunogens induces broader cellular immune responses and can be useful to minimize the degree of sequence dissimilarity among different virus strains.

[0025] Provided herein are proteins selected from the group consisting of: proteins comprising SEQ ID NO:2, proteins that are 95% identical over the entire length of the amino acid sequence of SEQ ID NO:2; fragments of SEQ ID NO:2; proteins that are 95% identical to a fragment of SEQ ID NO:2; SEQ ID NO:4, proteins that are 95% identical over the entire length of the amino acid sequence of SEQ ID NO:4; fragments of SEQ ID NO:4; proteins that are 95% identical to a fragment of SEQ ID NO:4 SEQ ID NO:6, proteins that are 95% identical over the entire length of the amino acid sequence of SEQ ID NO:6; fragments of SEQ ID NO:6; and proteins that are 95% identical to a fragment of SEQ ID NO:6.

[0026] Provided also herein is a protein selected from the group consisting of (a) SEQ ID NO:2; (b) a protein that is 95% identical over the entire length of the amino acid sequence of the full length sequence as set forth in SEQ ID NO:2; (c) an immunogenic fragment of SEQ ID NO:2 comprising 20 or more amino acids of SEQ ID NO:2; and (d) an immunogenic

fragment of a protein that is 95% identical over the entire length of the amino acid sequence of SEQ ID NO:2 comprising 20 or more amino acids.

[0027] Nucleic acid molecules comprising sequences that encode one or more protein molecules set forth above are also provided. In some embodiments, the nucleic acid molecule comprises a sequence selected from the group consisting of: SEQ ID NO:1; a nucleic acid sequence that is 95% identical over the entire length of the amino acid sequence of SEQ ID NO:1; a fragment of SEQ ID NO:1; a nucleic acid sequence that is 95% identical to a fragment of SEQ ID NO:1; SEQ ID NO:3; a nucleic acid sequence that is 95% identical over the entire length of the amino acid sequence of SEQ ID NO:3; a fragment of SEQ ID NO:3; a nucleic acid sequence that is 95% identical to a fragment of SEQ ID NO:3; SEQ ID NO:5; a nucleic acid sequence that is 95% identical over the entire length of the amino acid sequence of SEQ ID NO:5; a fragment of SEQ ID NO:5; and a nucleic acid sequence that is 95% identical to a fragment of SEQ ID NO:5.

[0028] Some aspects of the invention provide methods of inducing an immune response against HBV comprising the step of: administering to an individual HBV core antigen and/or HBV surface antigen molecules and/or compositions.

[0029] Additional aspects of the invention provide methods of protecting an individual against HBV infection. The methods comprise the step of: administering to said individual a prophylactically effective amount of a nucleic acid molecule comprising such nucleic acid sequence or compositions; wherein the nucleic acid sequence is expressed in cells of said individual and a protective immune response is induced against a protein encoded by said nucleic acid sequence.

[0030] In some aspects of the invention, methods are provided for treating an individual who has been infected by HBV. The methods comprise the step of: administering to said individual a therapeutically effective amount of such nucleic acid molecules and/or composition.

[0031] The present invention provides in another aspect a nucleic acid molecule comprising a coding sequence that encodes one or more proteins selected from the group consisting of: (a) a protein comprising SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16; (b) a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16; (c) an immunogenic fragment of a protein comprising SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16 that is at least 20 amino acids; and (d) an immunogenic fragment

of a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16 that is at least 20 amino acids.

[0032] The nucleic acid molecule can comprise one or more sequences selected from the group consisting of: (a) a nucleic acid sequence comprising SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15; (b) a nucleic acid sequence that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15; (c) fragments thereof that comprise a nucleic acid sequence encoding immunogenic fragments comprising at least 20 amino acids encoded by SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15; and (d) fragments thereof that comprise a nucleic acid sequence encoding immunogenic fragments comprising at least 20 amino acids of a protein that is 98% identical over the entire length of the amino acid sequence of a protein encoded by SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15. The nucleic acid molecule can be a plasmid. The nucleic acid molecule can be an expression vector and sequences encoding said one or more proteins are operably linked to regulatory elements. The nucleic acid molecule can be incorporated into a viral particle.

[0033] Some aspects of the invention provide a method of inducing an immune response against an HBV antigen comprising administering a nucleic acid molecule of the present invention to a subject.

[0034] Some aspects of the invention provide a method of protecting a subject from HBV infection comprising administering a nucleic acid molecule of present invention to the subject.

[0035] Some aspects of the invention provide a method of protecting a subject who has been diagnosed with HBV infection comprising administering a nucleic acid molecule of the present invention to the subject.

[0036] The present invention provides in yet another aspect a protein selected from the group consisting of: (a) SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16; (b) a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16; (c) an immunogenic fragment of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16 comprising 20 or more amino acids; and (d) an immunogenic fragment of a protein that is 98% identical over the entire length of the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16 comprising 20 or more amino acids.

[0037] Some aspects of the invention provide a vaccine useful for generating an immune response against HBV in a subject comprising: a nucleic acid molecule of the present invention, and an adjuvant molecule. The adjuvant can be IL-12, IL-15, IL-28, CTACK, TECK, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-18, IL-21, IL-31, IL-33 or a combination thereof; and in some embodiments, IL-12, IL-15, IL-28, or RANTES.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038] Figure 1 is a map showing the organization of the HBV genome which consists of four overlapping ORFs.

[0039] Figure 2 illustrates the organization and features of the HBV surface antigens.

[0040] Figure 3 illustrates IgELS and endoproteolytic cleavage sites in the consensus HBV surface antigen.

[0041] Figure 4 illustrates the long consensus HBV surface antigen (LHBs) and small consensus HBV surface antigen (SHBs).

[0042] Figure 5 is a map of the vector pGX1801 HepB – Mcore (SEQ ID NO:17).

[0043] Figure 6 is a map of the vector pGX1802 HepB pLHBs-A (SEQ ID NO:18).

[0044] Figure 7 is a map of the vector pGX1803 HepB pLHBs-C (SEQ ID NO:19).

[0045] Figure 8 is a map of the vector pGX1804 HepB pSHBs-A (SEQ ID NO:20).

[0046] Figure 9 is a map of the vector pGX1805 HepB SHBs-C (SEQ ID NO:21).

[0047] Figure 10 illustrates a phylogenetic analysis of HBcAg consensus sequence as compared to individual genotypes A, B, C, D, and E.

[0048] Figures 11A and 11B demonstrate results from pM Core expression experiments. Figure 11A shows results from *in vitro* translation protocol. Figure 11B shows results of a Western Blot.

[0049] Figure 12 is a graphic illustrating the plasmid map and sequence of pMCore.

[0050] Figure 13 shows a transcription/translation reaction using pMCore plasmid, in which the resulting MCore protein was immuno-precipitated with anti-HA monoclonal antibody and run on a SDS-PAGE gel.

[0051] Figure 14 shows detection of MCore protein in transiently transfected cells using a primary monoclonal HA tag antibody followed by detection with DyLight 594-labeled anti-rabbit secondary antibody. Hoechst stain was also used to fluorescently label cell nuclei.

Expression of MCore is mostly localized to the cytoplasm as shown by the staining outside the nucleus.

[0052] Figure 15 illustrates an immunization scheme. 4 mice were immunized intramuscularly with 30μg pMCore.

[0053] Figure 16 shows the enhanced magnitude of IFN-γ secretion in CD8+ and CD4+ T Cells from the spleens of Balb/C mice vaccinated with pM-Core.

[0054] Figure 17 shows the enhanced magnitude of IFN-γ secretion in CD8+ and CD4+ T Cells from the spleens of Balb/C mice vaccinated with pM-Core.

[0055] Figure 18 shows the enhanced magnitude of TNF-α secretion in CD8+ and CD4+ T Cells from the spleens of Balb/C mice vaccinated with pM-Core.

[0056] Figure 19 shows the enhanced magnitude of TNF-α secretion in CD8+ and CD4+ T Cells from the spleens of Balb/C mice vaccinated with pM-Core.

[0057] Figure 20 shows the enhanced magnitude of CD 107a secretion in CD8+ and CD4+ T Cells from the spleens of Balb/C mice vaccinated with pM-Core.

[0058] Figure 21 shows the enhanced magnitude of CD 107a secretion in CD8+ and CD4+ T Cells from the spleens of Balb/C mice vaccinated with pM-Core.

[0059] Figure 22 shows the average percent HBcAg-specific CD4 or CD8 IFN-γ^+, TNF-α^+ secreting cells from Balb/c mice immunized with the control pVAX or the consensus core antigen pMCore.

[0060] Figure 23 shows the average percent HBcAg-specific CD4 or CD8 double positive producing cells from Balb/c mice immunized with the control pVAX or the consensus core antigen pMCore.

[0061] Figure 24 shows interferon-gamma T cell response in liver from Balb/C mice vaccinated with pM-Core.

[0062] Figure 25 shows interferon-gamma T cell response in liver from Balb/C mice vaccinated with pM-Core.

[0063] Figure 26 shows Tumor Necrosis Factor-α T cell response in liver from Balb/C mice vaccinated with pM-Core.

[0064] Figure 27 shows Tumor Necrosis Factor-α T cell response in liver from Balb/C mice vaccinated with pM-Core.

[0065] Figure 28 shows the percent HBcAg-specific CD4 and CD8 IFN-γ^+, TNF-α^+ producing cells in the liver of Balb/c mice immunized with the control pVAX or the consensus core antigen pMCore.

[0066] Figure 29 shows the average percent HBcAg-specific CD4 or CD8 double positive producing cells in the liver of Balb/c mice immunized with the control pVAX or the consensus core antigen pMCore.

[0067] Figure 30 shows antigen-specific antibody producing splenocytes. The values are the means ± standard error of the mean. Significance was determined by Student's *t* test.

[0068] Figure 31 shows data from ELISPOT assays.

[0069] Figure 32 shows the frequency of HBcAg-specific IFN-γ spot forming units (SFU) per million splenocytes after stimulation of spleen cells from immunized mice.

[0070] Figure 33 shows data from experiments using CSFE labeled cells to compare elimination of peptide treated target cells *in vivo* by CD8 T cells in vaccinated and unvaccinated animals.

[0071] Figure 34 shows *in vivo* specific killing. Two groups of mice immunized with either pVax (control) or pMCore received CFSE-labeled target cells ($CFSE^{lo}$ pulsed with irrelevant peptide or $CFSE^{hi}$ pulsed with epitope-specific peptide) through the tail vain. CFSE-labeled cells were recovered and analysis by FACS was utilized to quantify percent killing.

[0072] Figure 35 shows a comparison of percent proliferation of CD3+CD4+ cells and CD3+CD8+ treated with pVax vector (control) or with plasmid pMCore which expresses HBV M-core.

[0073] Figure 36 shows the percent proliferation of CD4 and CD8 T cells.

[0074] Figures 37A and 37B shows a comparison of anti-HBV core IgG and IgA in serial dilution of sera from animals treated with pVax vector (control) or with plasmid pMCore which expresses HBV M-core.

[0075] Figure 38 shows the HBcAg-specific humoral immune response induced by pMCore in the sera and splenocytes. The values are the means ± standard error of the mean. Significance was determined by Student's *t* test.

[0076] Figure 39 shows the HBcAg-specific humoral immune response induced by pMCore in the sera and splenocytes. The values are the means ± standard error of the mean. Significance was determined by Student's *t* test.

[0077] Figure 40 shows percent TNF-α and IFN-γ from CD4+ and CD8+ spleen and liver cells.

[0078] Figure 41 shows data from experiments to determine if clearance induced by the immunized mice did effects liver by measuring ALT levels in sera.

[0079] Figure 42 shows immunostaining of liver sections taken three days after hydrodynamic injection of PBS, pMCore (HBcAg), or pNS3/4A (HCV NS3-4A) from naïve or mice that were immunized with pMcore is shown. Clearance is much higher for the pMCore-immunized liver as compared to the NS3/4a transfected control liver. PMcore or NS3/4A expression detected with an anti-HA antibody (white/light gray cells).

[0080] Figure 43 shows 3 days post transfection, in which cells were analyzed for degranulation marker expression, CD107a, and IFN-γ+ expression following stimulation with HBcAg peptides.

[0081] Figure 44 shows serum ALT levels at day 3, 6, and 12 post transfection were measured and showed no evidence of elevation in relevant vaccinated animals.

[0082] Figure 45 shows total cellular immune responses in monkeys immunized with the small (i.e., pMCore, pSHb A, and pSHb C), long (i.e., pMCore, pLHb A, and pLHb C), and long+IL-12 (i.e., pMCore, pLHb A, pLHb C, and prlIL-12) vaccines.

[0083] Figure 46 shows core-specific cellular immune responses in monkeys immunized with the small (i.e., pMCore, pSHb A, and pSHb C), long (i.e., pMCore, pLHb A, and pLHb C), and long+IL-12 (i.e., pMCore, pLHb A, pLHb C, and prlIL-12) vaccines.

[0084] Figure 47 shows surface antigen A-specific cellular immune responses in monkeys immunized with the small (i.e., pMCore, pSHb A, and pSHb C), long (i.e., pMCore, pLHb A, and pLHb C), and long+IL-12 (i.e., pMCore, pLHb A, pLHb C, and prlIL-12) vaccines.

[0085] Figure 48 shows data from ELISPOT assays surface antigen C-specific cellular immune responses in monkeys immunized with the small (i.e., pMCore, pSHb A, and pSHb C), long (i.e., pMCore, pLHb A, and pLHb C), and long+IL-12 (i.e., pMCore, pLHb A, pLHb C, and prlIL-12) vaccines.

[0086] Figure 49 shows total cellular immune responses in monkeys immunized with the small (i.e., pMCore, pSHb A, and pSHb C), long (i.e., pMCore, pLHb A, and pLHb C), and long+IL-12 (i.e., pMCore, pLHb A, pLHb C, and prlIL-12) vaccines.

[0087] Figure 50 shows a comparison of anti-HBV antibodies in serial dilution of sera from monkeys immunized with the small (i.e., pMCore, pSHb A, and pSHb C), long (i.e., pMCore, pLHb A, and pLHb C), and long+IL-12 (i.e., pMCore, pLHb A, pLHb C, and prlIL-12) vaccines.

[0088] Figure 51 shows a comparison of anti-HBV antibodies in serial dilution from animals immunized with the long+IL-12 (i.e., pMcore, pLHb A, pLHb C, and prlIL-12) vaccine.

[0089] Figure 52 shows HBV surface antigen DNA constructs and their expression. (A) Phylogenetic analysis of HBs consensus sequences of genotypes A and C as compared to natural HBs. The consensus sequences are indicated with asterisks. (B) Schematic diagram of HBs gene inserts in DNA plasmids. pLHBs and pSHBs represent large and small HBs protein antigens. The preS1, preS2 and major S proteins are linked with endoproteolytic cleavage sites. (C) Detection of pLHBs and pSHBs via intracellular staining of transfected RD cells with monoclonal antibody and polyclonal mouse sera.

[0090] Figure 53 shows that both pLHBs and SHBs elicited robust antibody and T cell responses in Balb/c. Five Balb/c mice per group were immunized in three different experiments and analyzed for their induction of both humoral and cellular responses (A) HBs-specific IgG responses measured one week after each immunization. Arrow indicates immunization time point. (B) Total IFN-γ responses induced by each constructs to synthetic preS1/S2 and major S peptides. (C) Characterization of HBs-specific immunodominant epitopes by using individual HBs peptides in 16 and 12 matrix pools of LHBs and SHBs respectively. This shows enhanced breadth of the cell mediated immune responses.

[0091] Figure 54 shows *in vivo* specific killing. Different groups of mice immunized with either pVax (control) or any of the four HBs constructs received CFSE-labeled target cells ($CFSE^{lo}$ pulsed with irrelevant peptide or $CFSE^{hi}$ pulsed with epitope-specific peptide) through the tail vain. CFSE-labeled cells were recovered and analysis by FACS (fluorescence-activated cell sorting) was utilized to quantify percentage of killing.

[0092] Figure 55 shows that HBc/HBs vaccine cocktail induces robust immune responses. (A) HBs-specific antibody responses from sera of mice immunized with HBs/HBc vaccine cocktail at different immunization time points (arrow). (B) IFN-γ responses after *ex vivo* stimulation of splenocytes from HBs/HBc cocktail immunized mice. Immunized mice responded to both core and surface antigen peptides.

[0093] Figure 56 shows that antigen-specific CD8 T cells induce antiviral cytokines. Intracellular staining of anti-viral cytokines after *ex-vivo* stimulation of splenocytes from balb/c mice from different immunized groups (Table 3). Lymphocytes were stained with antibodies to CD3, CD4, CD8, IFN-γ, TNF-α, IL-2 and CD107a. This data is a representative of 4 mice per group in 3 different experiments.

[0094] Figure 57 shows immunogenicity in non-human primates. Five Rhesus Macaques were immunized intramuscularly with pSHBs/pMCore, pLHBs/pMCore or pLHBs/pMCore + pIL-12 (Table 4) followed by electroporation. The monkeys received three immunizations.

(A) High antibody titers to HBs were observed after just 2 immunizations. (B) IFN-γ responses were measured once before the first immunization and 2 weeks following each immunization. (C) Many positive HBs matrix peptide pools were observed in most monkeys from the different groups confirming broad T cell responses in immunized macaques. (D) Intracellular staining (IFN-γ, TNF-α, IL-2) of PBMCs after the last immunization shows HBs and HBc-specific antiviral cytokine production in both CD4 and CD8.

[0095] Figures 58A-58D shows the safety of a therapeutic HBV vaccine in rhesus macaques. Samples were analyzed prior to vaccination (pre), after the third vaccination (3rd), prior to the fourth vaccination (pre4th) after the fourth vaccination (4th). There was an assay error for alkaline phosphatase after the third vaccination. The normal range for all measures is depicted by dashed lines. (A) Serum alkaline phosphatase. (B) Serum alanine aminotransferase (ALT) levels. (C) Serum aspartate aminotransferase (AST) levels. (D) Serum total bilirubin levels. (E) Serum creatinine levels. (F) Serum blood urea nitrogen levels.

[0096] Figures 59A-59D show the sequence annotation for the four HBs plasmids. The sequences for the IgE leader, endoproteolytic cleavage site and C-terminal HA tag are underlined. The four HBs plasmids are: Genotype A large surface protein (pLHBs-A) (A) and small surface protein (pSHBs-A) (B) and genotype C large surface protein (pLHBs-C) (C) and small surface protein (pSHBs-C) (D).

DETAILED DESCRIPTION

[0097] The present invention relates to highly optimized multivalent synthetic hepatitis B virus (HBV) immunogens that elicits both antibody and cell-mediated immunity against HBV surface (HBs) and HBV core (HBc) antigens. These plasmids, which encode the most prevalent genotypes of the virus, induce binding antibodies to HBs antigens and drive robust cell mediated immunity (CMI) to both HBc and HBs antigens. The plasmids independently elicited broad cross-reactive T cell responses and high titer antibody responses, as well as vaccine-induced cytotoxic activity. Plasmid cocktails containing both surface antigens induce strong and broad cellular immune response across multiple antigenic epitopes, in particular, broad cross reactivity between the A and C genotypes of the virus. Optimized plasmid cocktails encoding the HBs and HBc antigens induced strong humoral and cellular

responses targeting the S antigen and drove diverse multi-epitope responses involved with clearing infected hepatocytes

[0098] In particular, the present invention relates to vaccines for Hepatitis B virus (HBV) that can be used to protect an individual from HBV infection and to provide therapeutic benefits to an individual diagnosed with HBV infection. Such vaccines can also be used to induce an immune response against an HBV antigen. The HBV vaccine of the present invention includes one or more nucleic acid molecules. Particularly, the nucleic acid molecule encodes one or more consensus HBV core proteins. The consensus HBV core proteins are derived from the sequences of the core proteins from HBV genotypes A, B, C, D, and E, and therefore the consensus HBV core proteins are unique. The nucleic acid molecules can also encode one or more immunogenic fragments of the consensus HBV core proteins.

[0099] Additionally, the nucleic acid molecules can encode one or more consensus HBV surface antigens. The consensus HBV surface antigens are derived from sequences of the surface antigens from isolates of HBV genotype A, and therefore the consensus HBV surface antigens are unique. The consensus HBV surface antigens can also be derived from sequences of the surface antigens from isolates of HBV genotype C, resulting in unique HBV surface antigens. The nucleic acid molecules can also encode one or more immunogenic fragments of the consensus HBV surface antigens.

[00100] The vaccine of the present invention can include any combination of nucleic acid molecules encoding the consensus HBV core protein and/or nucleic acid molecules encoding the consensus HBV surface antigen. The vaccine can also include any combination of nucleic acid molecules encoding immunogenic fragments of the consensus HBV core proteins and/or nucleic acid molecules encoding immunogenic fragments of the consensus HBV surface antigens.

[00101] These combinations of the HBV core protein and HBV surface antigen surprisingly and unexpectedly induce a differential immune response to the HBV core protein and HBV surface antigen. In other words, the strength of the immune response to the HBV core protein and HBV surface antigen differs depending on the specific combination administered to a subject. Accordingly, any user of the vaccine of the present invention can design a vaccine that comprises a specific combination to induce a desired immune response in a subject administered such a designed vaccine. As such, any user can tailor the vaccine of the present invention to control the level of the immune response in the subject.

[00102] The vaccine of the present invention is widely applicable to multiple types of HBV because of the unique consensus sequences of the HBV core protein and HBV surface antigen.

[00103] The vaccine of the present invention can further include one or more nucleic acid molecules as described above and one or more proteins encoded by such nucleic acid molecules.

1. Definitions

[00104] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[00105] For recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the numbers 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[00106] "Adjuvant" as used herein means any molecule added to the DNA plasmid vaccines described herein to enhance the immunogenicity of the antigens encoded by the DNA plasmids and the encoding nucleic acid sequences described hereinafter.

[00107] "Antibody" as used herein means an antibody of classes IgG, IgM, IgA, IgD or IgE, or fragments, fragments or derivatives thereof, including Fab, F(ab')2, Fd, and single chain antibodies, diabodies, bispecific antibodies, bifunctional antibodies and derivatives thereof. The antibody can be an antibody isolated from the serum sample of mammal, a polyclonal antibody, affinity purified antibody, or mixtures thereof which exhibits sufficient binding specificity to a desired epitope or a sequence derived therefrom.

[00108] "Coding sequence" or "encoding nucleic acid" as used herein means the nucleic acids (RNA or DNA molecule) that comprise a nucleotide sequence which encodes a protein. The coding sequence can further include initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of an individual or mammal to which the nucleic acid is administered.

[00109] "Complement" or "complementary" as used herein means a nucleic acid can mean Watson-Crick (e.g., A-T/U and C-G) or Hoogsteen base pairing between nucleotides or nucleotide analogs of nucleic acid molecules.

[00110] "Consensus" or "consensus sequence" as used herein means a polypeptide sequence based on analysis of an alignment of multiple subtypes of a particular HBV antigen such as an HBV core or surface antigen. Nucleic acid sequences that encode a consensus polypeptide sequence can be prepared. Vaccines comprising proteins that comprise consensus sequences and/or nucleic acid molecules that encode such proteins can be used to induce broad immunity against multiple subtypes or serotypes of a particular HBV antigen.

[00111] "Electroporation," "electro-permeabilization," or "electro-kinetic enhancement" ("EP") as used interchangeably herein means the use of a transmembrane electric field pulse to induce microscopic pathways (pores) in a bio-membrane; their presence allows biomolecules such as plasmids, oligonucleotides, siRNA, drugs, ions, and water to pass from one side of the cellular membrane to the other.

[00112] "Fragment" as used herein with respect to nucleic acid sequences means a nucleic acid sequence or a portion thereof, that encodes a polypeptide capable of eliciting an immune response in a mammal that cross reacts with a full length wild type strain HBV antigen. The fragments can be DNA fragments selected from at least one of the various nucleotide sequences that encode protein fragments set forth below.

[00113] "Fragment" or "immunogenic fragment" with respect to polypeptide sequences means a polypeptide capable of eliciting an immune response in a mammal that cross reacts with a full length wild type strain HBV antigen. Fragments of consensus proteins can comprise at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or at least 95% of a consensus protein. In some embodiments, fragments of consensus proteins can comprise at least 20 amino acids or more, at least 30 amino acids or more, at least 40 amino acids or more, at least 50 amino acids or more, at least 60 amino acids or more, at least 70 amino acids or more, at least 80 amino acids or more, at least 90 amino acids or more, at least 100 amino acids or more, at least 110 amino acids or more, at least 120 amino acids or more, at least 130 amino acids or more, at least 140 amino acids or more, at least 150 amino acids or more, at least 160 amino acids or more, at least 170 amino acids or more, at least 180 amino acids or more, at least 190 amino acids or more, at least 200 amino acids or more, at least 210 amino acids or more, at least 220 amino acids or more, at least 230 amino acids or more, or at least 240 amino acids or more of a consensus protein.

[00114] As used herein, the term "genetic construct" refers to the DNA or RNA molecules that comprise a nucleotide sequence which encodes a protein. The coding sequence includes

initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the individual to whom the nucleic acid molecule is administered. As used herein, the term "expressible form" refers to gene constructs that contain the necessary regulatory elements operable linked to a coding sequence that encodes a protein such that when present in the cell of the individual, the coding sequence will be expressed.

[00115] The term "homology," as used herein, refers to a degree of complementarity. There can be partial homology or complete homology (i.e., identity). A partially complementary sequence that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially *homologous.*" When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially *homologous,*" as used herein, refers to a probe that can hybridize to a strand of the double-stranded nucleic acid sequence under conditions of low stringency. When used in reference to a single-stranded nucleic acid sequence, the term "substantially *homologous,*" as used herein, refers to a probe that can hybridize to (i.e., is the complement of) the single-stranded nucleic acid template sequence under conditions of low stringency.

[00116] "Identical" or "identity" as used herein in the context of two or more nucleic acids or polypeptide sequences, means that the sequences have a specified percentage of residues that are the same over a specified region. The percentage can be calculated by optimally aligning the two sequences, comparing the two sequences over the specified region, determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the specified region, and multiplying the result by 100 to yield the percentage of sequence identity. In cases where the two sequences are of different lengths or the alignment produces one or more staggered ends and the specified region of comparison includes only a single sequence, the residues of single sequence are included in the denominator but not the numerator of the calculation. When comparing DNA and RNA, thymine (T) and uracil (U) can be considered equivalent. Identity can be performed manually or by using a computer sequence algorithm such as BLAST or BLAST 2.0.

[00117] “Immune response” as used herein means the activation of a host’s immune system, e.g., that of a mammal, in response to the introduction of antigen such as an HBV

consensus antigen. The immune response can be in the form of a cellular or humoral response, or both.

[00118] "Nucleic acid" or "oligonucleotide" or "polynucleotide" as used herein means at least two nucleotides covalently linked together. The depiction of a single strand also defines the sequence of the complementary strand. Thus, a nucleic acid also encompasses the complementary strand of a depicted single strand. Many variants of a nucleic acid can be used for the same purpose as a given nucleic acid. Thus, a nucleic acid also encompasses substantially identical nucleic acids and complements thereof. A single strand provides a probe that can hybridize to a target sequence under stringent hybridization conditions. Thus, a nucleic acid also encompasses a probe that hybridizes under stringent hybridization conditions.

[00119] Nucleic acids can be single stranded or double stranded, or can contain portions of both double stranded and single stranded sequence. The nucleic acid can be DNA, both genomic and cDNA, RNA, or a hybrid, where the nucleic acid can contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine and isoguanine. Nucleic acids can be obtained by chemical synthesis methods or by recombinant methods.

[00120] "Operably linked" as used herein means that expression of a gene is under the control of a promoter with which it is spatially connected. A promoter can be positioned 5' (upstream) or 3' (downstream) of a gene under its control. The distance between the promoter and a gene can be approximately the same as the distance between that promoter and the gene it controls in the gene from which the promoter is derived. As is known in the art, variation in this distance can be accommodated without loss of promoter function.

[00121] "Promoter" as used herein means a synthetic or naturally-derived molecule which is capable of conferring, activating or enhancing expression of a nucleic acid in a cell. A promoter can comprise one or more specific transcriptional regulatory sequences to further enhance expression and/or to alter the spatial expression and/or temporal expression of same. A promoter can also comprise distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A promoter can be derived from sources including viral, bacterial, fungal, plants, insects, and animals. A promoter can regulate the expression of a gene component constitutively, or differentially with respect to cell, the tissue or organ in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as

physiological stresses, pathogens, metal ions, or inducing agents. Representative examples of promoters include the bacteriophage T7 promoter, bacteriophage T3 promoter, SP6 promoter, lac operator-promoter, tac promoter, SV40 late promoter, SV40 early promoter, RSV-LTR promoter, CMV IE promoter, SV40 early promoter or SV40 late promoter and the CMV IE promoter.

[00122] "Signal peptide" and "leader sequence" are used interchangeably herein and refer to an amino acid sequence that can be linked at the amino terminus of an HBV protein set forth herein. Signal peptides/leader sequences typically direct localization of a protein. Signal peptides/leader sequences used herein preferably facilitate secretion of the protein from the cell in which it is produced. Signal peptides/leader sequences are often cleaved from the remainder of the protein, often referred to as the mature protein, upon secretion from the cell. Signal peptides/leader sequences are linked at the N terminus of the protein.

[00123] "Stringent hybridization conditions" as used herein means conditions under which a first nucleic acid sequence (e.g., probe) will hybridize to a second nucleic acid sequence (e.g., target), such as in a complex mixture of nucleic acids. Stringent conditions are sequence-dependent and will be different in different circumstances. Stringent conditions can be selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm can be the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions can be those in which the salt concentration is less than about 1.0 M sodium ion, such as about 0.01-1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., about 10-50 nucleotides) and at least about 60°C for long probes (e.g., greater than about 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal can be at least 2 to 10 times background hybridization. Exemplary stringent hybridization conditions include the following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

[00124] "Substantially complementary" as used herein means that a first sequence is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the complement of a second sequence over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22,

23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 180, 270, 360, 450, 540, or more nucleotides or amino acids, or that the two sequences hybridize under stringent hybridization conditions.

[00125] "Substantially identical" as used herein means that a first and second sequence are at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 180, 270, 360, 450, 540 or more nucleotides or amino acids, or with respect to nucleic acids, if the first sequence is substantially complementary to the complement of the second sequence.

[00126] "Subtype" or "serotype": as used herein, interchangeably, and in reference to HBV, means genetic variants of an HBV such that one subtype is recognized by an immune system apart from a different subtype.

[00127] "Variant" used herein with respect to a nucleic acid means (i) a portion or fragment of a referenced nucleotide sequence; (ii) the complement of a referenced nucleotide sequence or portion thereof; (iii) a nucleic acid that is substantially identical to a referenced nucleic acid or the complement thereof; or (iv) a nucleic acid that hybridizes under stringent conditions to the referenced nucleic acid, complement thereof, or a sequences substantially identical thereto.

[00128] "Variant" with respect to a peptide or polypeptide that differs in amino acid sequence by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity. Variant can also mean a protein with an amino acid sequence that is substantially identical to a referenced protein with an amino acid sequence that retains at least one biological activity. A conservative substitution of an amino acid, i.e., replacing an amino acid with a different amino acid of similar properties (e.g., hydrophilicity, degree and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art. Kyte et al., J. Mol. Biol. 157:105-132 (1982). The hydropathic index of an amino acid is based on a consideration of its hydrophobicity and charge. It is known in the art that amino acids of similar hydropathic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydropathic indexes of ±2 are substituted. The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the

greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity. U.S. Patent No. 4,554,101, incorporated fully herein by reference. Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. Substitutions can be performed with amino acids having hydrophilicity values within ±2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties.

[00129] "Vector" as used herein means a nucleic acid sequence containing an origin of replication. A vector can be a viral vector, bacteriophage, bacterial artificial chromosome or yeast artificial chromosome. A vector can be a DNA or RNA vector. A vector can be a self-replicating extrachromosomal vector, and preferably, is a DNA plasmid.

2. Vaccines

[00130] The present invention is directed to a Hepatitis B vaccine. The Hepatitis B vaccine (HBV) can comprise a nucleic acid encoding a HBV core antigen, a HBV surface antigen, or a combination thereof; a HBV core antigen, a HBV surface antigen, or combination thereof, or a combination of (1) nucleic acid encoding a HBV core antigen and/or a HBV surface antigen, and (2) a HBV core antigen and/or a HBV surface antigen, or (3) combinations thereof. The HBV core antigen can comprise a consensus protein derived from the amino acid sequences of core proteins from multiple HBV genotypes. Similarly, the HBV surface antigen can comprise a consensus protein derived from the amino acid sequences of surface antigens from multiple HBV genotypes. Such consensus HBV core proteins and consensus HBV surface antigens are unique and have similarity to core and surface antigens, respectively, across multiple HBV genotypes. As such, the vaccine of the present invention is applicable to multiple types of HBV and is useful for widespread populations. Additionally, the vaccine of the present invention can be tailored to particular nucleic acids encoding consensus HBV core protein, consensus HBV surface protein, or a combination thereof. In other words, the vaccine of the present invention can be designed to control the level or strength of the immune response in the subject against one or more HBV serotypes.

[00131] The vaccine can be a DNA vaccine. DNA vaccines are disclosed in US Patent Nos. 5,593,972, 5,739,118, 5,817,637, 5,830,876, 5,962,428, 5,981,505, 5,580,859, 5,703,055, and 5,676,594, which are incorporated herein fully by reference. The DNA vaccine can further comprise elements or reagents that inhibit it from integrating into the chromosome.

[00132] The vaccine can be an RNA of the HBV core protein and/or the HBV surface antigen protein. The RNA vaccine can be introduced into the cell.

a. HBV Core antigen

[00133] The vaccine of the present invention can comprise a HBV core protein. The HBV core protein is a target for immune mediated viral clearance by inducing 1) cytotoxic T lymphocyte (CTL) responses, 2) T helper cell responses, and/or 3) B cell responses, or preferably all of the aforementioned, for cross presentation.

[00134] Table 2 shows the similarities across genotypes for core antigen from HBV-A, HBV-B, HBV-C, HBV-D and HBV-E genotypes with the consensus HBV core proteins, referred to in the chart as "HBV-M-core". For some embodiments, the HBV M Core construct was designed to have increased homologies for broad HBV core targets. Similarities in the genotypes for Core Antigen with designed M-Core construct - increased homologies for broad HBV core targets. All genotypes should be represented in a universal immune therapeutic vaccine for HBV.

Table 2 Percent Identities of HBV Core Proteins

Percent Identity									
Divergence		**1**	**2**	**3**	**4**	**5**	**6**		
	1		96.2	96.2	97.8	95.6	98.4		1 - HBV-A-ConCore
	2	3.9		100	95.6	93.4	96.7		2 - HBV-B-ConCore
	3	3.9	0		95.6	93.4	96.7		3 - HBV-C-ConCore
	4	2.2	4.5	4.5		97.8	97.8		4 - HBV-D-ConCore
	5	4.5	6.9	6.9	2.2		95.6		5 - HBV-E-ConCore
	6	1.7	3.4	3.4	2.2	4.5			6 - HBV-M-Core
		1	2	3	4	5	6		

[00135] The antigen can comprise core protein epitopes that make them particularly effective as immunogens against which anti-HBV immune responses can be induced. The HBV antigen can comprise the full length translation product, a variant thereof, a fragment thereof or a combination thereof.

[00136] The HBV core antigen can comprise a consensus protein. The HBV consensus core antigen induces antigen-specific T-cell and high titer antibody responses both systemically and in the liver. As such, a protective immune response is provided to the liver by vaccines comprising the HBV consensus core antigen. Accordingly, any user can design a vaccine of the present invention to include an HBV consensus core antigen to provide immune-based therapy and/or protection to the liver.

[00137] Particularly in the induced immune response, the HBV consensus core antigen stimulates splenocytes, specifically CD4+ cells and CD8+ cells, to secrete or produce similar amounts of interferon-gamma (INF-γ), but different amounts of tumor necrosis factor-alpha (TNF-α). Interestingly, the induced immune response differs in the spleen and liver. In the spleen, more CD8+ cells produce both INF-γ and TNF-α than CD4+ cells, however in the liver, the opposite occurs. In other words, more CD4+ cells produce both INF-γ and TNF-α than CD8+ cells in the liver. Additionally, in the liver, more antigen-specific IgA than IgG is produced, and the protective immune response surprisingly and unexpectedly includes an antigen-specific CTL response, which does not cause liver damage. Accordingly, a vaccine of the present invention comprising an HBV consensus core antigen can be delivered in the periphery to establish an antigen-specific targeting the liver to clear or eliminate HBV infected cells without causing damage to or inflammation of the liver.

[00138] The nucleic acid sequences encoding the consensus HBV core proteins are SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5. SEQ ID NO:1 encodes an HBV consensus core protein. SEQ ID NO:3 encodes the HBV consensus core protein linked to an IgE leader. SEQ ID NO:5 encodes the HBV consensus core protein linked to the IgE leader and an HA tag.

[00139] Some embodiments relate to nucleic acid sequences encoding proteins identical or homologous to the HBV consensus protein, immunogenic fragment of the HBV consensus protein, and immunogenic fragments of homologous proteins. Such nucleic acid molecules that encode immunogenic proteins that have up to 95% identity over the entire length of the amino acid sequence of a consensus sequence, up to 96% identity over the entire length of the amino acid sequence of a consensus sequence, up to 96% identity over the entire length of the

amino acid sequence of a consensus sequence, up to 97% identity over the entire length of the amino acid sequence of a consensus sequence, up to 98% identity over the entire length of the amino acid sequence of a consensus sequence and up to 99% can be provided. Likewise, nucleic acid sequences encoding the immunogenic fragments set forth herein and the immunogenic fragments of proteins identical to the proteins set forth herein are also provided.

[00140] Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 95% homology to the nucleic acid coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 96% homology to the nucleic acid coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 97% homology to the nucleic acid coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 98% homology to the nucleic acid coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 99% homology to the nucleic acid coding sequences herein. In some embodiments, the nucleic acid molecules with coding sequences disclosed herein that are homologous to a coding sequence of a consensus protein disclosed herein include sequences encoding an IgE leader sequence linked to the 5' end of the coding sequence encoding the homologous protein sequences disclosed herein.

[00141] Some embodiments relate to nucleic acid sequences encoding proteins with a particular percent identity over the entire length of the amino acid sequence of the full length HBV core consensus protein, immunogenic fragment of the HBV core consensus protein, and immunogenic fragments of HBV core consensus proteins. Such nucleic acid molecules that encode immunogenic proteins that have up to 80% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 85% identity over the entire length of the amino acid sequence of a full length consensus sequence, up to 90% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 91% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 92% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 93% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 94% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 95% identity over the entire length of the amino acid

sequence of a full length HBV core consensus sequence, up to 96% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 97% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 98% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence, up to 99% identity over the entire length of the amino acid sequence of a full length HBV core consensus sequence can be provided. Likewise, nucleic acid sequences encoding the immunogenic fragments set forth herein and the immunogenic fragments of proteins with similar percent identities as indicated above to the HBV core proteins set forth herein are also provided.

[00142] Some embodiments relate to nucleic acid molecules that encode immunogenic protein that have 80% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic protein that have 83% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 85% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 90% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 91% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 92% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 93% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 94% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 95% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid

molecules that encode immunogenic proteins that have 96% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 97% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 98% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 99% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV core protein coding sequences herein. In some embodiments, the nucleic acid molecules with coding sequences disclosed herein that are homologous to a coding sequence of a consensus HBV core protein disclosed herein include sequences encoding an IgE leader sequence linked to the 5' end of the coding sequence encoding the homologous protein sequences disclosed herein.

[00143] In some embodiments, the nucleic acid sequence is free of coding sequence that encodes a leader sequence. In some embodiments, the nucleic acid sequence is free of coding sequence that encodes the IgE leader.

[00144] Some embodiments relate to fragments of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5. Fragments can be at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5. Fragments can be at least 95%, at least 96%, at least 97% at least 98% or at least 99% identical to fragments of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5. Fragments can be at least 80%, at least 85%, at least 90% at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to fragments of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5. In some embodiments, fragments include sequences that encode a leader sequence, such as for example, an immunoglobulin leader, such as the IgE leader. In some embodiments, fragments are free of coding sequences that encode a leader sequence. In some embodiments, fragments are free of coding sequences that encode a leader sequence, such as for example, the IgE leader.

[00145] Furthermore, the amino acid sequence of the consensus HBV core protein is SEQ ID NO:2. The amino acid sequence of the consensus HBV core protein linked to an IgE leader is SEQ ID NO:4. Particularly, in SEQ ID NO:4, the IgE leader is linked to the amino terminus of the consensus HBV core protein. The amino acid sequence of the consensus HBV core protein linked to the IgE leader and an HA tag is SEQ ID NO:6. In SEQ ID NO:6, the IgE leader and HA tag are linked to the amino and carboxy termini, respectively, of the consensus HBV core protein.

[00146] Some embodiments relate to proteins that are homologous to SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have 95% homology to the consensus protein sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have 96% homology to the consensus protein sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.. Some embodiments relate to immunogenic proteins that have 97% homology to the consensus protein sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.. Some embodiments relate to immunogenic proteins that have 98% homology to the consensus protein sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.. Some embodiments relate to immunogenic proteins that have 99% homology to the consensus protein sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

[00147] Some embodiments relate to proteins that are identical to SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 80% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 85% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 90% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 91% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 92% identical over the entire length of the amino acid sequence

of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 93% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 94% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 95% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 96% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 97% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 98% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 99% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

[00148] In some embodiments, the protein is free of a leader sequence. In some embodiments, the protein is free of the IgE leader. Fragments of consensus proteins can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of a consensus protein. Immunogenic fragments of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 can be provided. Immunogenic fragments can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%,

at least 98% or at least 99% of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. In some embodiments, fragments include a leader sequence, such as for example, an immunoglobulin leader, such as the IgE leader. In some embodiments, fragments are free of a leader sequence. In some embodiments, fragments are free of a leader sequence, such as for example, the IgE leader.

[00149] Immunogenic fragments of proteins with amino acid sequences homologous to immunogenic fragments of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 can be provided. Such immunogenic fragments can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of proteins that are 95% homologous to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. Some embodiments relate to immunogenic fragments that have 96% homology to the immunogenic fragments of consensus protein sequences herein. Some embodiments relate to immunogenic fragments that have 97% homology to the immunogenic fragments of consensus protein sequences herein. Some embodiments relate to immunogenic fragments that have 98% homology to the immunogenic fragments of consensus protein sequences herein. Some embodiments relate to immunogenic fragments that have 99% homology to the immunogenic fragments of consensus protein sequences herein. In some embodiments, fragments include a leader sequence, such as for example, an immunoglobulin leader, such as the IgE leader. In some embodiments, fragments are free of a leader sequence. In some embodiments, fragments are free of a leader sequence, such as for example, the IgE leader.

[00150] Immunogenic fragments of proteins with amino acid sequences identical to immunogenic fragments of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6 can be provided. Such immunogenic fragments can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of proteins that are 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical over the entire length of the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. In some embodiments, fragments include a leader sequence, such as for example, an immunoglobulin leader, such as the IgE leader. In some embodiments, fragments are free of

a leader sequence. In some embodiments, fragments are free of a leader sequence, such as for example, the IgE leader.

[00151] As referred to herein with regard to linking a signal peptide or leader sequence to the N terminus of a protein, the signal peptide/leader sequence replaces the N terminal methionine of a protein which is encoded by the start codon of the nucleic acid sequence than encodes the protein without a signal peptide coding sequences. Thus for example, SEQ ID NO:4 is SEQ ID NO:2 with the signal peptide/leader sequence linked at the N terminal of SEQ ID NO:2, i.e. SEQ ID NO:4 is a protein comprising a signal peptide linked to the N terminus of SEQ ID NO:2. The first residue in SEQ ID NO:2, "Xaa", is typically methionine when no signal peptide is present. However, proteins that comprise signal peptides linked to SEQ ID NO:2, such as SEQ ID NO:4, replace the residue 1 methionine at Xaa with the residue that links the signal peptide to the protein. Accordingly, the N terminal residue of SEQ ID NO:2 can be anything but if it is encoded by an initiation sequence it is methionine. The linkage of the signal peptide/leader sequence at the N terminal of SEQ ID NO:2 typically eliminates the N terminal methionine. As used herein, it is intended that SEQ ID NO:4 comprises SEQ ID NO:2 with a signal peptide/leader sequence linked at the N terminal of SEQ ID NO:2 notwithstanding the elimination of the N terminus Xaa residue of SEQ ID NO:2. Similarly, the coding sequences for SEQ ID NO:4 comprise coding sequences for SEQ ID NO:2 with coding sequences for a signal peptide/leader sequence linked to the 5' end of the coding sequences encoding SEQ ID NO:2. The initiation codon can be the "nnn" in the coding sequences for SEQ ID NO:2 but it is eliminated when the coding sequences for a signal peptide/leader sequence linked to the 5' end of the coding sequences encoding SEQ ID NO:2. As used herein, it is intended that coding sequences for SEQ ID NO:4 comprises coding sequences for SEQ ID NO:2 with coding sequences for a signal peptide/leader sequence linked at the 5' end of the coding sequence of SEQ ID NO:2 where nnn occurs. Thus, for example, it is intended that SEQ ID NO:3 comprises SEQ ID NO:1 with coding sequences for a signal peptide/leader sequence linked at the 5' end of SEQ ID NO:1, in place of the nnn. In some embodiments, the nnn is an initiation codon at the 5' end of SEQ ID NO:1.

b. HBV Surface Antigen

[00152] The vaccine can comprise a HBV surface antigen. Provided herein are HBV surface antigens capable of eliciting an immune response in a mammal against one or more

HBV serotypes. The surface antigens can comprise surface protein epitopes that make them particularly effective as immunogens against which anti-HBV immune response can be induced. The HBV surface antigen can comprise the full length translation product, a variant thereof, a fragment thereof, or a combination thereof.

[00153] The HBV surface antigen can comprise a consensus protein. Consensus HBV surface antigens were generated from the sequences of surface antigens from primary isolates of either HBV genotype A or C. Particularly, the consensus HBV surface antigens include S protein or the combination of S protein, pre-S2, and pre-S1 (Figures 3 and 4). Endoproteolytic cleavage sites were introduced into the consensus HBV surface antigens to provide for proper protein folding and better CTL processing. The codon usage in the consensus HBV surface antigens was modified to reflect the codon bias of human genes. Additionally, regions of very high (e.g., greater than 80 percent) or very low (e.g., less than 30 percent) GC content were avoided, as where cis-acting motifs such as internal TATA-boxes, repetitive sequences, and structured sequences. A Kozak sequence was introduced into the consensus HBV surface antigens to increase translational initiation and an IgE leader sequence was added to increase protein expression.

[00154] The nucleic acid sequence encoding the consensus HBV surface antigens are SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:15. SEQ ID NO:9 encodes a long consensus HBV surface antigen protein derived from genotype A and including S protein, pre-S2, and pre-S1 (LHBs-A). SEQ ID NO:11 encodes a long consensus HBV surface antigen protein derived from genotype C and including S protein, pre-S2, and pre-S1 (LHBs-C). SEQ ID NO:13 encodes a short consensus HBV surface antigen protein derived from genotype A and including S protein (SHBs-A). SEQ ID NO:15 encodes a short consensus HBV surface antigen protein derived from genotype C and including S protein (SHBs-C).

[00155] Some embodiments relate to nucleic acid sequences encoding proteins identical over the entire length of the amino acid sequence of the consensus HBV surface antigens, immunogenic fragments of the consensus HBV surface antigens, and immunogenic fragments of identical proteins. Thus, nucleic acid molecules that encode immunogenic proteins that have up to 95% identity over the entire length of the amino acid sequence of a consensus HBV surface antigen sequence, up to 96% identity over the entire length of the amino acid sequence of a consensus HBV surface antigen sequence, up to 96% identity over the entire length of the amino acid sequence of a consensus HBV surface antigen sequence,

up to 97% identity over the entire length of the amino acid sequence of a consensus HBV surface antigen sequence, up to 98% identity over the entire length of the amino acid sequence of a consensus HBV surface antigen sequence, and up to 99% identity over the entire length of the amino acid sequence of a consensus HBV surface antigen sequence can be provided. Likewise, nucleic acid sequences encoding the immunogenic fragments set forth herein and the immunogenic fragments of proteins identical over the entire length of the amino acid sequence of the HBV surface antigen proteins set forth herein are also provided.

[00156] Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 95% identity over the entire length of the amino acid sequence of the nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 96% identity over the entire length of the amino acid sequence of the nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 97% identity over the entire length of the amino acid sequence of the nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 98% identity over the entire length of the amino acid sequence of the nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 99% identity over the entire length of the amino acid sequence of the nucleic acid HBV surface antigen coding sequences herein. In some embodiments, the nucleic acid molecules with coding sequences disclosed herein that are identical over the entire length of the amino acid sequence of a coding sequence of a consensus HBV surface antigen protein disclosed herein include sequences encoding an IgE leader sequence linked to the 5' end of the coding sequence encoding the identical protein sequences disclosed herein.

[00157] Some embodiments relate to nucleic acid sequences encoding proteins with a particular percent identity over the entire length of the amino acid sequence of the full length HBV surface antigen consensus protein, immunogenic fragment of the HBV surface antigen consensus protein, and immunogenic fragments of HBV surface antigen consensus proteins. Such nucleic acid molecules that encode immunogenic proteins that have up to 80% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 85% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 90% identity over the entire

length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 91% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 92% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 93% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 94% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 95% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 96% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 97% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 98% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence, up to 99% identity over the entire length of the amino acid sequence of a full length HBV surface antigen consensus sequence can be provided. Likewise, nucleic acid sequences encoding the immunogenic fragments set forth herein and the immunogenic fragments of proteins with similar percent identities as indicated above to the HBV surface antigen proteins set forth herein are also provided.

[00158] Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 80% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 83% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 85% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 90% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 91% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 92% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some

embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 93% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 94% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 95% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 96% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 97% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 98% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. Some embodiments relate to nucleic acid molecules that encode immunogenic proteins that have 99% identity over the entire length of the amino acid sequence of the full length nucleic acid HBV surface antigen coding sequences herein. In some embodiments, the nucleic acid molecules with coding HBV surface antigen sequences disclosed herein that are identical over the entire length of the amino acid sequence of a coding sequence of a consensus HBV surface antigen protein disclosed herein include sequences encoding an IgE leader sequence linked to the 5' end of the coding sequence encoding the identical protein sequences disclosed herein.

[00159] In some embodiments, the nucleic acid sequence is free of coding sequence that encodes a leader sequence. In some embodiments, the nucleic acid sequence is free of coding sequence that encodes the IgE leader.

[00160] Some embodiments relate to fragments of SEQ ID NO:9, SEQ ID NO: 11, SEQ ID NO:13, and SEQ ID NO:15. Fragments can be at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15. Fragments can be at least 95%, at least 96%,

at least 97% at least 98% or at least 99% identical to fragments of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15. Fragments can be at least 80%, at least 85%, at least 90% at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to fragments of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO: 13 or SEQ ID NO:15. In some embodiments, fragments include sequences that encode a leader sequence, such as for example, an immunoglobulin leader, such as the IgE leader. In some embodiments, fragments are free of coding sequences that encode a leader sequence. In some embodiments, fragments are free of coding sequences that encode a leader sequence, such as the IgE leader.

[00161] Furthermore, the amino acid sequences of the consensus HBV surface antigens, LHBs-A, LHBs-C, SHBs-A, and SHBs-C, are SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:16, respectively.

[00162] Proteins can be identical to SEQ ID NO:10, SEQ ID NO: 12, SEQ ID NO:14, or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have 95% identity over the entire length of the amino acid sequence of the consensus protein sequences herein. Some embodiments relate to immunogenic proteins that have 96% identity over the entire length of the amino acid sequence of the consensus protein sequences herein. Some embodiments relate to immunogenic proteins that have 97% identity over the entire length of the amino acid sequence of the consensus protein sequences herein. Some embodiments relate to immunogenic proteins that have 98% identity over the entire length of the amino acid sequence of the consensus protein sequences herein. Some embodiments relate to immunogenic proteins that have 99% identity over the entire length of the amino acid sequence of the consensus protein sequences herein.

[00163] Some embodiments relate to proteins that are identical to SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 80% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 85% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 90% identical over the entire length of the amino acid sequence of the full length consensus amino

acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 91% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 92% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 93% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 94% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 95% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 96% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 97% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 98% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16. Some embodiments relate to immunogenic proteins that have an amino acid sequence that is 99% identical over the entire length of the amino acid sequence of the full length consensus amino acid sequences as set forth in SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO: 14 or SEQ ID NO:16.

[00164] Fragments of consensus proteins can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at

least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of a consensus protein. Immunogenic fragments of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:16 can be provided. Immunogenic fragments can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16.

[00165] Immunogenic fragments of proteins with amino acid sequences identical to immunogenic fragments of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16can be provided. Such immunogenic fragments can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of proteins that are 95% identical over the entire length of the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16.

[00166] Immunogenic fragments of proteins with amino acid sequences identical to immunogenic fragments of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16 can be provided. Such immunogenic fragments can comprise at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or at least 55% at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of proteins that are 95% identical over the entire length of the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16. Some embodiments relate to immunogenic fragments that have 96% identity to the immunogenic fragments of consensus protein sequences herein. Some embodiments relate to immunogenic fragments that have 97% identity to the immunogenic fragments of consensus protein sequences herein. Some embodiments relate to immunogenic fragments that have 98% identity to the immunogenic fragments of consensus protein sequences herein. Some embodiments relate to immunogenic fragments that have 99% identity to the immunogenic fragments of consensus protein sequences herein.

c. Combination of HBV Core and Surface Antigens

[00167] The vaccine can comprise a combination of the HBV core and surface antigens as described above. This combination of HBV antigens is capable of eliciting an immune response in a mammal against one or more HBV serotypes. The vaccine can be designed or tailored to have a particular combination of HBV antigens, which in turn provides the ability to control the level or strength of an immune response in the mammal.

[00168] The combinations can comprise one or more nucleic acids encoding (1) HBV-M-Core, LHBs-A, SHBs-A, LHBs-C, and SHBs-C; (2) HBV-M-Core, LHBs-A, SHBs-A, and LHBs-C; (3) HBV-M-Core, LHBs-A, and SHBs-A; (4) HBV-M-Core and LHBs-A; (5) HBV-M-Core, SHBs-A, LHBs-C, and SHBs-C; (6) HBV-M-Core, LHBs-C, and SHBs-C; (7) HBV-M-Core and SHBs-C; (8) HBV-M-Core, LHBs-A, LHBs-C, and SHBs-C; (9) HBV-M-core, LHBs-A, and SHBs-C; (10) HBV-M-Core and SHBs-C; (11) HBV-M-Core, LHBs-A, SHBs-A, and SHBs-C; (12) HBV-M-Core, LHBs-A, and SHBs-C; (13) HBV-M-Core, SHBs-A, and SHBs-C; (14) LHBs-A, SHBs-A, LHBs-C, and SHBs-C; (15) LHBs-A, SHBs-A, and LHBs-C; (16) LHBs-A and SHBs-A; (17) SHBs-A, LHBs-C, and SHBs-C; (18) LHBs-C and SHBs-C; (19) LHBs-A, LHBs-C, and SHBs-C; (20) LHBs-A and SHBs-C; (21) LHBs-A, SHBs-A, and SHBs-C; (22) LHBs-A and SHBs-C; (23) SHBs-A and SHBs-C; (24) HBV-M-Core and SHBs-A; (25) HBV-M-Core, LHBs-A and LHBs-C; or (26) LHBs-A and LHBs-C.

[00169] An exemplary embodiment relates to a vaccine including one or more nucleic acids encoding HBV-M-Core, LHBs-A, and LHBs-C. Another exemplary embodiment relates to vaccine including one or more nucleic acids encoding HBV-M-Core, SHBs-A, and SHBs-C. Yet another exemplary embodiment relates to a vaccine including one or more nucleic acids encoding HBV-M-Core, LHBs-A, and LHBs-C, and adjuvant such as IL-12.

[00170] The combinational vaccine also comprises one or more consensus HBV core protein and/or HBV surface antigen protein in the form of one or more protein subunits, one or more killed viral particles comprising one or more consensus HBV core protein and/or consensus HBV surface antigen protein, or one or more attenuated viral particles comprising one or more consensus HBV core protein and/or HBV surface antigen protein. The attenuated vaccine can be attenuated live vaccines, killed vaccines and vaccines that use recombinant vectors to deliver foreign genes that encode one or more consensus HBV core protein and/or HBV surface antigen protein, and well as subunit and glycoprotein vaccines. Examples of attenuated live vaccines, those using recombinant vectors to deliver foreign antigens, subunit

vaccines and glycoprotein vaccines are described in U.S. Patent Nos.: 4,510,245; 4,797,368; 4,722,848; 4,790,987; 4,920,209; 5,017,487; 5,077,044; 5,110,587; 5,112,749; 5,174,993; 5,223,424; 5,225,336; 5,240,703; 5,242,829; 5,294,441; 5,294,548; 5,310,668; 5,387,744; 5,389,368; 5,424,065; 5,451,499; 5,453,3 64; 5,462,734; 5,470,734; 5,474,935; 5,482,713; 5,591,439; 5,643,579; 5,650,309; 5,698,202; 5,955,088; 6,034,298; 6,042,836; 6,156,319 and 6,589,529, which are each incorporated herein by reference.

d. Vaccine Constructs and Plasmids

[00171] The vaccine can comprise nucleic acid constructs or plasmids that encode the HBV core proteins, the HBV surface antigens, and combinations of the HBV core proteins/surface antigens. Provided herein are genetic constructs that can comprise a nucleic acid sequence that encodes the HBV core antigen disclosed herein including consensus protein sequences, sequences homologous to consensus protein sequences, fragments of consensus protein sequences and sequences homologous to fragments of consensus protein sequences. Additionally, provided herein are genetic constructs that can comprise a nucleic acid sequence that encodes the HBV surface antigen disclosed herein including consensus protein sequences, sequences homologous to consensus protein sequences, fragments of consensus protein sequences and sequences homologous to fragments of consensus protein sequences. The genetic construct can be present in the cell as a functioning extrachromosomal molecule. The genetic construct can be a linear minichromosome including centromere, telomeres or plasmids or cosmids.

[00172] The genetic construct can also be part of a genome of a recombinant viral vector, including recombinant adenovirus, recombinant adenovirus associated virus and recombinant vaccinia. The genetic construct can be part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which live in cells.

[00173] The genetic constructs can comprise regulatory elements for gene expression of the coding sequences of the nucleic acid. The regulatory elements can be a promoter, an enhancer an initiation codon, a stop codon, or a polyadenylation signal.

[00174] The nucleic acid sequences can make up a genetic construct that can be a vector. The vector can be capable of expressing an antigen in the cell of a mammal in a quantity effective to elicit an immune response in the mammal. The vector can be recombinant. The vector can comprise heterologous nucleic acid encoding the antigen. The vector can be a plasmid. The vector can be useful for transfecting cells with nucleic acid encoding an antigen,

which the transformed host cell is cultured and maintained under conditions wherein expression of the antigen takes place.

[00175] Coding sequences can be optimized for stability and high levels of expression. In some instances, codons are selected to reduce secondary structure formation of the RNA such as that formed due to intramolecular bonding.

[00176] The vector can comprise heterologous nucleic acid encoding an antigen and can further comprise an initiation codon, which can be upstream of the antigen coding sequence, and a stop codon, which can be downstream of the antigen coding sequence. The initiation and termination codon can be in frame with the antigen coding sequence. The vector can also comprise a promoter that is operably linked to the antigen coding sequence. The promoter operably linked to the antigen coding sequence can be a promoter from simian virus 40 (SV40), a mouse mammary tumor virus (MMTV) promoter, a human immunodeficiency virus (HIV) promoter such as the bovine immunodeficiency virus (BIV) long terminal repeat (LTR) promoter, a Moloney virus promoter, an avian leukosis virus (ALV) promoter, a cytomegalovirus (CMV) promoter such as the CMV immediate early promoter, Epstein Barr virus (EBV) promoter, or a Rous sarcoma virus (RSV) promoter. The promoter can also be a promoter from a human gene such as human actin, human myosin, human hemoglobin, human muscle creatine, or human metalothionein. The promoter can also be a tissue specific promoter, such as a muscle or skin specific promoter, natural or synthetic. Examples of such promoters are described in US patent application publication no. US20040175727, the contents of which are incorporated herein in its entirety.

[00177] The vector can also comprise a polyadenylation signal, which can be downstream of the HBV core protein coding sequence. The polyadenylation signal can be a SV40 polyadenylation signal, LTR polyadenylation signal, bovine growth hormone (bGH) polyadenylation signal, human growth hormone (hGH) polyadenylation signal, or human β-globin polyadenylation signal. The SV40 polyadenylation signal can be a polyadenylation signal from a pCEP4 vector (Invitrogen, San Diego, CA).

[00178] The vector can also comprise an enhancer upstream of the consensus HBV core protein coding sequence or the consensus HBV surface antigen protein coding sequence. The enhancer can be necessary for DNA expression. The enhancer can be human actin, human myosin, human hemoglobin, human muscle creatine or a viral enhancer such as one from CMV, HA, RSV or EBV. Polynucleotide function enhances are described in U.S. Patent

Nos. 5,593,972, 5,962,428, and WO94/016737, the contents of each are fully incorporated by reference.

[00179] The vector can also comprise a mammalian origin of replication in order to maintain the vector extrachromosomally and produce multiple copies of the vector in a cell. The vector can be pVAX1, pCEP4 or pREP4 from Invitrogen (San Diego, CA), which can comprise the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region, which can produce high copy episomal replication without integration. The vector can be pVAX1 or a pVax1 variant with changes such as the variant plasmid described herein. The variant pVax1 plasmid is a 2998 basepair variant of the backbone vector plasmid pVAX1 (Invitrogen, Carlsbad CA). The CMV promoter is located at bases 137-724. The T7 promoter/priming site is at bases 664-683. Multiple cloning sites are at bases 696-811. Bovine GH polyadenylation signal is at bases 829-1053. The Kanamycin resistance gene is at bases 1226-2020. The pUC origin is at bases 2320-2993.

[00180] Based upon the sequence of pVAX1 available from Invitrogen, the following mutations were found in the sequence of pVAX1 that was used as the backbone for plasmids 1-6 set forth herein:

[00181] C>G241 in CMV promoter

[00182] C>T1942 backbone, downstream of the bovine growth hormone polyadenylation signal (bGHpolyA)

[00183] A> -2876 backbone, downstream of the Kanamycin gene

[00184] C>T3277 in pUC origin of replication (Ori) high copy number mutation (see Nucleic Acid Research 1985)

[00185] G>C 3753 in very end of pUC Ori upstream of RNASeH site

[00186] Base pairs 2, 3 and 4 are changed from ACT to CTG in backbone, upstream of CMV promoter.

[00187] The backbone of the vector can be pAV0242. The vector can be a replication defective adenovirus type 5 (Ad5) vector.

[00188] The vector can also comprise a regulatory sequence, which can be well suited for gene expression in a mammalian or human cell into which the vector is administered. The consensus HBV coding sequence can comprise a codon, which can allow more efficient transcription of the coding sequence in the host cell.

[00189] The vector can be pSE420 (Invitrogen, San Diego, Calif.), which can be used for protein production in Escherichia coli (E. coli). The vector can also be pYES2 (Invitrogen,

San Diego, Calif.), which can be used for protein production in Saccharomyces cerevisiae strains of yeast. The vector can also be of the MAXBAC™ complete baculovirus expression system (Invitrogen, San Diego, Calif.), which can be used for protein production in insect cells. The vector can also be pcDNA I or pcDNA3 (Invitrogen, San Diego, Calif.), which may be used for protein production in mammalian cells such as Chinese hamster ovary (CHO) cells. The vector can be expression vectors or systems to produce protein by routine techniques and readily available starting materials including Sambrook et al., Molecular Cloning and Laboratory Manual, Second Ed., Cold Spring Harbor (1989),which is incorporated fully by reference.

[00190] In some embodiments the vector can comprise the nucleic acid sequence of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, or SEQ ID NO:21. SEQ ID NO:17 encodes a consensus HBV core protein and SEQ ID NOS:18-21 encodes a consensus HBV surface antigen. The vector maps of SEQ ID NOS:17-21 are shown in Figures 5-9, respectively.

e. Pharmaceutical Compositions of the Vaccine

[00191] The vaccine can be in the form of a pharmaceutical composition. The pharmaceutical composition can comprise the vaccine.

[00192] The pharmaceutical compositions can comprise about 5 nanograms to about 10 mg of the vaccine DNA. In some embodiments, pharmaceutical compositions according to the present invention comprise about 25 nanogram to about 5 mg of vaccine DNA. In some embodiments, the pharmaceutical compositions contain about 50 nanograms to about 1 mg of DNA. In some embodiments, the pharmaceutical compositions contain about 0.1 to about 500 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 1 to about 350 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 5 to about 250 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 10 to about 200 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 15 to about 150 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 20 to about 100 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 25 to about 75 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 30 to about 50 micrograms of DNA. In some embodiments, the pharmaceutical compositions contain about 35 to about 40 micrograms of DNA. In some embodiments, the

pharmaceutical compositions contain about 100 to about 200 microgram DNA. In some embodiments, the pharmaceutical compositions comprise about 10 microgram to about 100 micrograms of DNA. In some embodiments, the pharmaceutical compositions comprise about 20 micrograms to about 80 micrograms of DNA. In some embodiments, the pharmaceutical compositions comprise about 25 micrograms to about 60 micrograms of DNA. In some embodiments, the pharmaceutical compositions comprise about 30 nanograms to about 50 micrograms of DNA. In some embodiments, the pharmaceutical compositions comprise about 35 nanograms to about 45 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 100 to about 200 microgram DNA.

[00193] In some embodiments, pharmaceutical compositions according to the present invention comprise at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nanograms of DNA of the vaccine. In some embodiments, the pharmaceutical compositions can comprise at least 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95,100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 605, 610, 615, 620, 625, 630, 635, 640, 645, 650, 655, 660, 665, 670, 675, 680, 685, 690, 695, 700, 705, 710, 715, 720, 725, 730, 735, 740, 745, 750, 755, 760, 765, 770, 775, 780, 785, 790, 795, 800, 805, 810, 815, 820, 825, 830, 835, 840, 845, 850, 855, 860, 865, 870, 875, 880, 885, 890, 895. 900, 905, 910, 915, 920, 925, 930, 935, 940, 945, 950, 955, 960, 965, 970, 975, 980, 985, 990, 995 or 1000 micrograms of DNA of the vaccine. In some embodiments, the pharmaceutical composition can comprise at least 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg or more of DNA of the vaccine.

[00194] In other embodiments, the pharmaceutical composition can comprise up to and including 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nanograms of DNA of the vaccine. In some embodiments, the pharmaceutical composition can comprise

up to and including 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95,100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, 350, 355, 360, 365, 370, 375, 380, 385, 390, 395, 400, 405, 410, 415, 420, 425, 430, 435, 440, 445, 450, 455, 460, 465, 470, 475, 480, 485, 490, 495, 500, 605, 610, 615, 620, 625, 630, 635, 640, 645, 650, 655, 660, 665, 670, 675, 680, 685, 690, 695, 700, 705, 710, 715, 720, 725, 730, 735, 740, 745, 750, 755, 760, 765, 770, 775, 780, 785, 790, 795, 800, 805, 810, 815, 820, 825, 830, 835, 840, 845, 850, 855, 860, 865, 870, 875, 880, 885, 890, 895. 900, 905, 910, 915, 920, 925, 930, 935, 940, 945, 950, 955, 960, 965, 970, 975, 980, 985, 990, 995, or 1000 micrograms of DNA of the vaccine. In some embodiments, the pharmaceutical composition can comprise up to and including 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 mg of DNA of the vaccine.

[00195] The pharmaceutical composition can further comprise other agents for formulation purposes according to the mode of administration to be used. In cases where pharmaceutical compositions are injectable pharmaceutical compositions, they are sterile, pyrogen free and particulate free. An isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation.

[00196] The vaccine can further comprise a pharmaceutically acceptable excipient. The pharmaceutically acceptable excipient can be functional molecules as vehicles, adjuvants, carriers, or diluents. The pharmaceutically acceptable excipient can be a transfection facilitating agent, which can include surface active agents, such as immune-stimulating complexes (ISCOMS), Freunds incomplete adjuvant, LPS analog including monophosphoryl lipid A, muramyl peptides, quinone analogs, vesicles such as squalene and squalene, hyaluronic acid, lipids, liposomes, calcium ions, viral proteins, polyanions, polycations, or nanoparticles, or other known transfection facilitating agents.

[00197] The transfection facilitating agent is a polyanion, polycation, including poly-L-glutamate (LGS), or lipid. The transfection facilitating agent is poly-L-glutamate, and more preferably, the poly-L-glutamate is present in the vaccine at a concentration less than 6 mg/ml. The transfection facilitating agent can also include surface active agents such as

immune-stimulating complexes (ISCOMS), Freunds incomplete adjuvant, LPS analog including monophosphoryl lipid A, muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid can also be used administered in conjunction with the genetic construct. In some embodiments, the DNA vector vaccines can also include a transfection facilitating agent such as lipids, liposomes, including lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture (see for example W09324640), calcium ions, viral proteins, polyanions, polycations, or nanoparticles, or other known transfection facilitating agents. Preferably, the transfection facilitating agent is a polyanion, polycation, including poly-L-glutamate (LGS), or lipid. Concentration of the transfection agent in the vaccine is less than 4 mg/ml, less than 2 mg/ml, less than 1 mg/ml, less than 0.750 mg/ml, less than 0.500 mg/ml, less than 0.250 mg/ml, less than 0.100 mg/ml, less than 0.050 mg/ml, or less than 0.010 mg/ml.

[00198] The pharmaceutically acceptable excipient can be an adjuvant. The adjuvant can be other genes that are expressed in alternative plasmid or are delivered as proteins in combination with the plasmid above in the vaccine. The adjuvant can be selected from the group consisting of: α-interferon(IFN-α), β-interferon (IFN-β), γ-interferon, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), cutaneous T cell-attracting chemokine (CTACK), epithelial thymus-expressed chemokine (TECK), mucosae-associated epithelial chemokine (MEC), IL-12, IL-15, MHC, CD80,CD86 including IL-15 having the signal sequence deleted and optionally including the signal peptide from IgE. The adjuvant can be IL-12, IL-15, IL-28, CTACK, TECK, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-18, IL-21, IL-31, IL-33 or a combination thereof. In an exemplary embodiment, the adjuvant is IL-12.

[00199] Other genes which can be useful adjuvants include those encoding: MCP-1, MIP-la, MIP-1p, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, pl50.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, CD40L, vascular growth factor, fibroblast growth factor, IL-7, nerve growth factor, vascular endothelial growth factor, Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, Caspase ICE, Fos, c-jun, Sp-1, Ap-1, Ap-2, p38, p65Rel, MyD88, IRAK, TRAF6, IkB, Inactive NIK, SAP K, SAP-1, JNK, interferon response genes, NFkB, Bax, TRAIL, TRAILrec, TRAILrecDRC5, TRAIL-R3, TRAIL-R4,

RANK, RANK LIGAND, Ox40, Ox40 LIGAND, NKG2D, MICA, MICB, NKG2A, NKG2B, NKG2C, NKG2E, NKG2F, TAP1, TAP2 and functional fragments thereof.

f. Methods of Vaccine Delivery

[00200] Provided herein is a method for delivering the pharmaceutical formulations for providing genetic constructs and proteins of the HBV core protein and/or HBV surface antigen which comprise epitopes that make them particular effective immunogens against which an immune response to HBV viral infections can be induced. The method of delivering the vaccine, or vaccination, can be provided to induce a therapeutic and/or prophylactic immune response. The vaccination process can generate in the mammal an immune response against a plurality of HBV genotypes. The vaccine can be delivered to an individual to modulate the activity of the mammal's immune system and enhance the immune response. The delivery of the vaccine can be the transfection of the HA antigen as a nucleic acid molecule that is expressed in the cell and delivered to the surface of the cell upon which the immune system recognizes and induces a cellular, humoral, or cellular and humoral response. The delivery of the vaccine can be used to induce or elicit an immune response in mammals against a plurality of HBV viruses by administering to the mammals the vaccine as discussed herein.

[00201] Upon delivery of the vaccine to the mammal, and thereupon the vector into the cells of the mammal, the transfected cells will express and secrete consensus HBV core protein and consensus HBV surface antigen. These secreted proteins, or synthetic antigens, will be recognized as foreign by the immune system, which will mount an immune response that can include: antibodies made against the antigens, and T-cell response specifically against the antigen. In some examples, a mammal vaccinated with the vaccines discussed herein will have a primed immune system and when challenged with an HBV viral strain, the primed immune system will allow for rapid clearing of subsequent HBV viruses, whether through the humoral, cellular, or both. The vaccine can be delivered to an individual to modulate the activity of the individual's immune system thereby enhancing the immune response.

[00202] Methods of delivering the DNA of a vaccine are described in U.S. Patent Nos. 4,945,050 and 5,036,006, both of which are incorporated herein in their entirety by reference.

[00203] The vaccine can be administered to a mammal to elicit an immune response in a mammal. The mammal can be human, non-human primate, cow, pig, sheep, goat, antelope,

bison, water buffalo, bovids, deer, hedgehogs, elephants, llama, alpaca, mice, rats, or chicken, and preferably human, cow, pig, or chicken.

g. Delivery of Vaccine with Adjuvants

[00204] The pharmaceutical compositions, preferably vaccines described herein, can be administered in combination with proteins or genes encoding adjuvants, which can include: α-interferon(IFN-α), β-interferon (IFN-β), γ-interferon, IL-12, IL-15, IL-28, CTACK, TECK, platelet derived growth factor (PDGF), TNFα, TNFβ, GM-CSF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, MCP-1, MIP-la, MIP-1p, IL-8, RANTES, L-selectin, P-selectin, E-selectin, CD34, GlyCAM-1, MadCAM-1, LFA-1, VLA-1, Mac-1, pl50.95, PECAM, ICAM-1, ICAM-2, ICAM-3, CD2, LFA-3, M-CSF, G-CSF, IL-4, mutant forms of IL-18, CD40, CD40L, vascular growth factor, fibroblast growth factor, IL-7, nerve growth factor, vascular endothelial growth factor, Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6, Caspase ICE, Fos, c-jun, Sp-1, Ap-1, Ap-2, p38, p65Rel, MyD88, IRAK, TRAF6, IkB, Inactive NIK, SAP K, SAP-1, JNK, interferon response genes, NFkB, Bax, TRAIL, TRAILrec, TRAILrecDRC5, TRAIL-R3, TRAIL-R4, RANK, RANK LIGAND, Ox40, Ox40 LIGAND, NKG2D, MICA, MICB, NKG2A, NKG2B, NKG2C, NKG2E, NKG2F, TAP1, or TAP2, or functional fragments thereof. In an exemplary embodiment, the adjuvant is IL-12.

h. Method of Generating an Immune Response with the Vaccine

[00205] The vaccine can be used to generate an immune response in a mammal, including therapeutic or prophylactic immune response. The immune response can generate antibodies and/or killer T cells which are directed to the HBV core antigen, HBV surface antigen, or the combination thereof. Such antibodies and T cells can be isolated.

[00206] Some embodiments provide methods of generating immune responses against HBV core proteins, HBV surface antigen proteins, and the combination thereof, which comprise administering to an individual the vaccine. Some embodiments provide methods of prophylactically vaccinating an individual against HBV infection, which comprise administering the vaccine. Some embodiments provide methods of therapeutically vaccinating an individual that has been infected with HBV, which comprise administering the

vaccine. Diagnosis of HBV infection prior to administration of the vaccine can be done routinely.

i. Method of Treatment with the Vaccine

[00207] The vaccine can be used to generate an immune response in a mammal that is protective of the liver. The immune response can generate an antigen-specific CTL response that does not cause damage to or inflammation of the liver. In some embodiments, the vaccine can be delivered to the periphery to establish an antigen-specific immune response targeting the liver to clear or eliminate HBV infected cells without damaging or causing inflammation of the liver. In some embodiments, treatment can include delivery of a vaccine comprising an HBV consensus core antigen to the periphery to establish an antigen-specific immune response targeting the liver to clear or eliminate HBV infected cells without causing damage to or inflammation of the liver.

3. Routes of Administration

[00208] The vaccine or pharmaceutical composition can be administered by different routes including orally, parenterally, sublingually, transdermally, rectally, transmucosally, topically, via inhalation, via buccal administration, intrapleurally, intravenous, intraarterial, intraperitoneal, subcutaneous, intramuscular, intranasal intrathecal, and intraarticular or combinations thereof. For veterinary use, the composition can be administered as a suitably acceptable formulation in accordance with normal veterinary practice. The veterinarian can readily determine the dosing regimen and route of administration that is most appropriate for a particular animal. The vaccine can be administered by traditional syringes, needleless injection devices, "microprojectile bombardment gone guns", or other physical methods such as electroporation ("EP"), "hydrodynamic method", or ultrasound.

[00209] The vector of the vaccine can be delivered to the mammal by several well known technologies including DNA injection (also referred to as DNA vaccination) with and without *in vivo* electroporation, liposome mediated, nanoparticle facilitated, recombinant vectors such as recombinant adenovirus, recombinant adenovirus associated virus and recombinant vaccinia. The HBV antigen can be delivered via DNA injection and along with *in vivo* electroporation.

a. Electroporation

[00210] The vaccine or pharmaceutical composition can be administered by electroporation. Administration of the vaccine via electroporation can be accomplished using electroporation devices that can be configured to deliver to a desired tissue of a mammal a pulse of energy effective to cause reversible pores to form in cell membranes, and preferable the pulse of energy is a constant current similar to a preset current input by a user. The electroporation device can comprise an electroporation component and an electrode assembly or handle assembly. The electroporation component can include and incorporate one or more of the various elements of the electroporation devices, including: controller, current waveform generator, impedance tester, waveform logger, input element, status reporting element, communication port, memory component, power source, and power switch. The electroporation can be accomplished using an *in vivo* electroporation device, for example CELLECTRA® EP system (Inovio Pharmaceuticals, Inc., Blue Bell, PA) or Elgen electroporator (Inovio Pharmaceuticals, Inc.) to facilitate transfection of cells by the plasmid.

[00211] Examples of electroporation devices and electroporation methods that can facilitate delivery of the DNA vaccines of the present invention, include those described in U.S. Patent No. 7,245,963 by Draghia-Akli, et al., U.S. Patent Pub. 2005/0052630 submitted by Smith, et al., the contents of which are hereby incorporated by reference in their entirety. Other electroporation devices and electroporation methods that can be used for facilitating delivery of the DNA vaccines include those provided in co-pending and co-owned U.S. Patent Application, Serial No. 11/874072, filed October 17, 2007, which claims the benefit under 35 USC 119(e) to U.S. Provisional Applications Ser. Nos. 60/852,149, filed October 17, 2006, and 60/978,982, filed October 10, 2007, all of which are hereby incorporated in their entirety.

[00212] U.S. Patent No. 7,245,963 by Draghia-Akli, et al. describes modular electrode systems and their use for facilitating the introduction of a biomolecule into cells of a selected tissue in a body or plant. The modular electrode systems can comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The biomolecules are then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical

pulse facilitates the introduction of the biomolecule into the cell between the plurality of electrodes. The entire content of U.S. Patent No. 7,245,963 is hereby incorporated by reference.

[00213] U.S. Patent Pub. 2005/0052630 submitted by Smith, et al. describes an electroporation device which can be used to effectively facilitate the introduction of a biomolecule into cells of a selected tissue in a body or plant. The electroporation device comprises an electro-kinetic device ("EKD device") whose operation is specified by software or firmware. The EKD device produces a series of programmable constant-current pulse patterns between electrodes in an array based on user control and input of the pulse parameters, and allows the storage and acquisition of current waveform data. The electroporation device also comprises a replaceable electrode disk having an array of needle electrodes, a central injection channel for an injection needle, and a removable guide disk. The entire content of U.S. Patent Pub. 2005/0052630 is hereby incorporated by reference.

[00214] The electrode arrays and methods described in U.S. Patent No. 7,245,963 and U.S. Patent Pub. 2005/0052630 can be adapted for deep penetration into not only tissues such as muscle, but also other tissues or organs. Because of the configuration of the electrode array, the injection needle (to deliver the biomolecule of choice) is also inserted completely into the target organ, and the injection is administered perpendicular to the target issue, in the area that is pre-delineated by the electrodes. The electrodes described in U.S. Patent No. 7,245,963 and U.S. Patent Pub. 2005/005263 are preferably 20 mm long and 21 gauge.

[00215] Additionally, contemplated in some embodiments that incorporate electroporation devices and uses thereof, there are electroporation devices that are those described in the following patents: US Patent 5,273,525 issued December 28, 1993, US Patents 6,110,161 issued August 29, 2000, 6,261,281 issued July 17, 2001, and 6,958,060 issued October 25, 2005, and US patent 6,939,862 issued September 6, 2005. Furthermore, patents covering subject matter provided in US patent 6,697,669 issued February 24, 2004, which concerns delivery of DNA using any of a variety of devices, and US patent 7,328,064 issued February 5, 2008, drawn to method of injecting DNA are contemplated herein. The above-patents are incorporated by reference in their entirety.

4. Method of Preparing the Vaccine

[00216] Provided herein are methods for preparing the DNA plasmids that comprise the vaccines discussed herein. The DNA plasmids, after the final subcloning step into the

mammalian expression plasmid, can be used to inoculate a cell culture in a large scale fermentation tank, using known methods in the art.

[00217] The DNA plasmids for use with the EP devices of the present invention can be formulated or manufactured using a combination of known devices and techniques, but preferably they are manufactured using an optimized plasmid manufacturing technique that is described in a US published application no. 20090004716, which was filed on May 23, 2007. In some examples, the DNA plasmids used in these studies can be formulated at concentrations greater than or equal to 10 mg/mL. The manufacturing techniques also include or incorporate various devices and protocols that are commonly known to those of ordinary skill in the art, in addition to those described in U.S. Serial No. 60/939792, including those described in a licensed patent, US Patent No. 7,238,522, which issued on July 3, 2007. The above-referenced application and patent, US Serial No. 60/939,792 and US Patent No. 7,238,522, respectively, are hereby incorporated in their entirety.

EXAMPLES

[00218] The present invention is further illustrated in the following examples. It should be understood that these examples, while indicating preferred embodiments of the invention, is given by way of illustration only. From the above discussion and the examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

EXAMPLE 1

HBV Core Protein

[00219] A consensus HBV core protein, also referred to as HBV modified, MCore or M-core construct, was designed from epitope sequences from HBV genotypes A, B, C, D and E. HBV core protein sequences from these genotypes were selected for inclusion in a construction of a consensus core that would induce immunity against a broad range of genotypes, thus providing a universal vaccine for HBV. In some embodiments,

modifications of the M-core construct included addition of an IgE leader sequence. In some embodiments, the M-core protein is encoded using codon optimization and RNA optimization for enhanced expression.

1. Construction and expression of consensus core antigen

[00220] An HBV genotype A, B, C, D, and E Core consensus nucleotide sequence was constructed by generating consensus sequences of core genes for each genotype and then generating a consensus sequence of all five genotype consensuses, thus a bias toward heavily sequenced genotypes was avoided. Additionally, the sequences were collected from different countries to avoid sampling bias towards heavily sequenced genotypes. The sequences were aligned using clustal X software to develop the final HBcAg consensus sequence. As shown in Figure 10, there was an observed relative closeness of the multi-genotype consensus HBcAg sequence to all sampled sequences from different genotypes.

[00221] After the consensus sequence was generated, several modifications were performed to increase the antigen expression levels from plasmids. Particularly, a highly efficient IgE leader sequence and a C-terminal HA tag were added, and the construct was RNA and codon optimized. This resulted in a nucleic acid sequence encoding M-core sequence with IgE leader and HA Tag (SEQ ID NO:5), which was digested with EcoRI and NotI, and cloned into the expression vector pVAX (Invitrogen) under the control of the cytomegalovirus immediate-early promoter. The resulting construct was then named as pMCore (SEQ ID NO:17).

[00222] *In vitro* expression tests were done using the pMCore construct and pVAX, which was used as a control. The results showing positive expression are depicted in the gel images shown in Figures 11A and 11B.

[00223] Additionally, the HBcAg protein was expressed by transfecting pMCore DNA plasmid containing the core gene of hepatitis B (Figure 12). Expression of pMCore was detected using TNT® Quick Coupled Expression of Transcription/Translation System containing ^{35}S-methionine (Promega, Madison, WI). The synthesized gene product was immuno-precipitated using an anti-HA monoclonal antibody targeting an encoded HA epitope, Clone HA-7 (Sigma-Aldrich). The immuno-precipitated protein was electrophoresed on a 12% SDS-PAGE gel and subsequently fixed and dried. The synthesized protein with incorporation of radioactive ^{35}S was detected by autoradiography. This *in vitro* translation assay on lysate showed detectable HBcAg at an expected molecular weight of 28 kDa (Figure 13).

[00224] The expression was further confirmed using anti-HA tagged monoclonal antibody by immunofluorescent assay. Rhabdomyosarcoma (RD) cell lines were transfected with pMCore using TURBOFECT (Thermo Scientific) according to the manufacturer's guidelines. The cells were first fixed with 2% formaldehyde and then assayed for protein expression. The fixed cells were incubated with rabbit monoclonal HA tag (Invitrogen) diluted in 'primary standard solution' (0.1% BSA, 0.2% saponin, 0.02% sodium azide) for an hour at room temperature. The cells were subsequently incubated with DyLight 594-labeled anti-rabbit secondary antibody (Thermo Scientific) for 20 minutes at room temperature. Confocal imaging was used to visualize HBcAg in the cytoplasm and around the nucleus of transfected RD cells as shown in Figure 14. The expression pattern confirmed that a DNA plasmid carrying the consensus core gene could be highly expressed in different cells *in vitro*. Specifically, images were obtained using a Zeiss Axiovert 100 inverted confocal microscope. Analysis and quantification of florescence intensities were conducted using Image J software (NIH, Rockville, MD).

2. Immunization of mice

[00225] C57BL/6 transgenic mice were separated into two groups of four mice each and using electroporation immunized three times with 20 μg DNA at biweekly intervals (group 1 – pVAX vector control; group 2 pM-Core). Mice were immunized on Day 0, Day 14, Day 28 and sacrificed on Day 35. Spleens, liver and sera were harvested from sacrificed the animals.

[00226] Additionally, and to evaluate the generation of T and B cell immune responses, Balb/c mice were immunized and measured for both responses in various peripheral tissues. Mice received three intramuscular immunizations of 30μg of pMCore or pVax followed by electroporation as depicted in the immunization scheme (Figure 15). Particularly, six to eight week old female Balb/c mice were purchased from Jackson Laboratories. Animals were maintained in accordance with the National Institutes of Health and the University of Pennsylvania Institutional Care and Use Committee (IACUC) guidelines. For DNA immunization studies, eight animals were divided into two groups. Each animal in the immunized group received a total of three immunizations of 30 μg of pMCore two weeks apart in the tibialis anterior (TA) muscle. Each immunization was accompanied by *in vivo* electroporation with the CELLECTRA adaptive constant current electroporation device (Inovio Pharmaceuticals, Blue Bell, PA). Two 0.2 Amp constant current square-wave pulses were delivered through a triangular 3-electrode array consisting of 26-gauge solid stainless

steel electrodes completely inserted into muscle. Each pulse was 52 milliseconds in length with a 1 second delay between pulses.

[00227] *In vivo* studies of Balb/C mice strains indicated enhancement in the magnitude of secretion of tumor necrosis factor (TNF-α), interferon gamma T-cell (IFN-γ) and the CD107a in the CD8 and CD4 T-cells taken from the spleen. Figures 16 and 17 demonstrate that vaccination of Balb/C mice with pM-Core enhanced the magnitude of IFN-γ secretion in CD8+ and CD4+ T Cells from the spleens. Figures 18 and 19 demonstrate that vaccination of Balb/C mice with pM-Core enhanced the magnitude of TNF-α secretion in CD8+ and CD4+ T Cells from the spleens. Figures 20 and 21 demonstrate that vaccination of Balb/C mice with pM-Core enhanced the magnitude of CD 107a secretion in CD8+ and CD4+ T Cells from the spleens.

[00228] In additional experiments, IFN-γ and TNF-α secretion were examined in T cells. To harvest splenocytes for these additional experiments, mice were sacrificed one week after the last immunization and spleens were harvested and placed in R10 media (RPMI media supplemented with 10% FBS and 1x Antibiotic-Antimycotic). The spleens were individually crushed, strained with a 40μM cell strainer, and treated with 1mL ACK lysis buffer for 5 min to lysis erythrocytes. The splenocytes were resuspended in a complete R10 media and used for further immunological assays.

[00229] A subset of splenocytes was resuspended in R10 media at a concentration of 10^7 per mL and 100μL was plated onto a 96 well round bottom plate. 100μL of media containing pMCore pooled peptides or 10 ng/ml PMA (Sigma, St. Louis, MO, USA) and 500ng/ml ionomycin (Calbiochem, Novabiochem, La Jolla, CA, USA) mix as a positive control or 0.1% dimethyl sulfoxide (Sigma, St. Louis, MO, USA) as a negative control. All wells contained 5 μL/mL of two protein transport inhibitors, brefeldin A (GolgiPlug) and monensin (Golgistop) (All from BD Bioscience). The cells were incubated at 37°C in 5% CO_2 for 5 hrs and stained with LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) for 10min at 37°C. Extracellular staining was performed using antibodies specific to mouse CD3, CD4 and CD8. Splenocytes were then permeabilized and washed using BD CYTOFIX/CYTOPERM and PERM/WASH (BD Bioscience), respectively.

[00230] *Intracellular Cytokine Staining.*

[00231] Intracellular cytokines were then stained with antibodies to mouse Interferon-gamma and Tumor Necrosis Factor-alpha. A subset of lymphocytes was resuspended in R10 media at a concentration of 10^7 per mL and 100 μL was plated onto a 96 well round bottom

plate. 100 µL of media containing pMCore pooled peptides or 10 ng/ml PMA (Sigma, St. Louis, MO, USA) and 500 ng/ml ionomycin (Calbiochem, Novabiochem, La Jolla, CA, USA) mix as a positive control or 0.1% dimethyl sulfoxide (Sigma, St. Louis, MO, USA) as a negative control. All wells contained 5 µL/mL of two protein transport inhibitors, brefeldin A (GolgiPlug) and monensin (Golgistop) (All from BD Bioscience). The cells were incubated at 37°C in 5% CO_2 for 5 hrs and stained with LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen) for 10 min at 37°C. Extracellular staining was performed using antibodies specific to mouse CD3, CD4 and CD8. Spleenocytes were then permeabilized and washed using BD Cytofix/Cytoperm™ and Perm/Wash™ (BD Bioscience) respectively. The cells were then stained intracellularly with antibodies to Interferon-gamma and Tumor Necrosis Factor- alpha, Interluekin-2 and Lysosomal-associated membrane protein 1.

[00232] Conjugated anti-mouse antibodies were used during the extracellular and intracellular staining including: CD3-Phycoerythrin/Cy7 (PE/Cy7), CD4-peridinin chlorophyll protein (PerCP), CD8-allophycocyanin (APC), IFN-γ-Alexa Fluor 700, TNF-a-fluorescein isothiocyanate (FITC) and IL-2-phycoerythryin cyanine (PE) (all from BD Biosciences, San Jose, CA).

[00233] The average HBcAg-specific IFN-γ T cell response induced was robust at 2000 (± 210) SFU per million splenocytes. Interestingly, intracellular staining of stimulated splenocytes revealed that both $CD4^+$ and $CD8^+$ cells produce almost the similar amount of antigen-specific IFN-γ, 0.74 and 0.94, respectively, but different levels of TNF-α with about 0.3% and 1.5% of the $CD4^+$ and $CD8^+$ cells, respectively (Figure 22). A similar trend was observed with cells that were double positive for both cytokines. There were lesser double positive $CD4^+$ cells, about 0.2%, in the spleen than double positive $CD8^+$ cells, which averages 0.7% (Figure 23).

[00234] HBV specific T-cell migration to the liver was also demonstrated in animals administered the pM-Core DNA vaccine. Targeting HBV core antigen specific T cells with high frequency and effector function to the liver is a goal for the development of an HBV immune therapy. Following immunization, animals were sacrificed and their livers were removed and HBV specific effector T cell migration to the liver was determined. The results show that the pM-Core vaccine drives effector T cells to the liver *in vivo*. Figures 24 and 25 demonstrate interferon-γ T cell liver response, and Figures 26 and 27 demonstrate Tumor Necrosis Factor-α liver immune response, and the elevated response that results from vaccination with pM-Core.

[00235] The M-core consensus immunogen encoded by the pM-core DNA construct drives strong balanced CD4+/CD8+ T cell immune responses. Induced T cells traffic to the liver at high frequency and exhibit the correct effector phenotype for immune clearance post HBV infection supporting further development of this immune therapeutic vaccine.

[00236] The cytokine producing capabilities of intrahepatic antigen-specific T cells after DNA immunization was also examined. Each liver was perfused by directly injecting 1mL of PBS into the hepatic vein of each mouse. Particularly, livers were harvested, crushed and resuspended in 5mL of 44% isotonic Percoll. The mixtures were underlied with 3mL 66% isotonic Percoll and centrifuged for 20 minutes at 2000rpm for gradient separation. Lymphocytes were collected and washed in 10 mL R10 and treated with ACK lysis buffer as necessary. Both CD4 and CD8 T cells isolated from the liver produce IFN-γ and TNF-α when stimulated *in vitro* with HBcAg peptide (Figures 28 and 29). While the CD4 T cells showed a high percentage of double producers, the CD8 showed little to no IFN-γ+TNF-α+ producing cells. Instead, a majority of the CD8 T cells produced only IFN-γ or TNF-α. An enrichment of HBcAg-specific CD4 T cells in the liver was observed, which was opposite to that of the spleen. The percent HBcAg-specific CD4 T double positive in the resting liver were comparable to that observed in the spleen. Moreover, peripheral CD8 T cells were confirmed to be better double producers than liver resident CD8 T cells. It was also observed that antibody-producing capabilities of liver resident B cells from immunized mice. Interestingly, the liver as a mucosal organ produced higher antigen-specific IgA than IgG (Figure 30), an observation that has not been previously been studied.

[00237] Figure 31 shows Cellular Immune responses Induced by pM-Core using an Enzyme-linked immunosorbent spot (ELISPOT) assay. Splenocytes were stimulated with two pools of 15-mer peptides spanning the entire length of pMCore and over lapping by 8 amino acids. 200,000 splenocytes in R10 media were plated in a 96 well IFN-γ capture antibody (R&D system) coated plate and stimulated overnight in the presence of a specific peptide pool at 37°C in 5% CO_2. Cells were washed out and plates were incubated overnight with biotinylated anti-mouse IFN-γ detection antibody (R&D system). Streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt and nitro blue tetrazolium chloride were subsequently used to develop spots. Spots were counted using an automated ELISPOT reader (CTL Limited). As shown in Figure 31, immunization with pMCore could induce strong cellular immune responses. The average HBcAg-specific IFN-γ T cell responses were about 2000 (± 210) SFU per million splenocytes.

[00238] The ELISPOT assay was used to further examine IFN-γ. Particularly, splenocytes were stimulated with two pools of 15-mer peptides spanning the entire length of HBcAg and over lapping by 8 amino acids. There were 33 total individual peptides, which were pooled randomly with the first 17 peptides going into pool 1 and last 16 in pool 2. The IgELs and HA tag were excluded to make the peptide as close to the natural antigen as possible. 200,000 splenocytes in R10 media were plated in a 96 well IFN-γ capture antibody (R&D system) coated plate and stimulated overnight in the presence of a specific peptide pool at 37°C in 5% CO_2. Cells were washed out and plates were incubated overnight with biotinylated anti-mouse IFN-γ detection antibody (R&D system). Streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt and nitro blue tetrazolium chloride were subsequently used to develop spots. Spots were counted using an automated ELISPOT reader (CTL Limited). It was observed that one week after the final immunization, pMCore immunized mice showed evidence of strong HBcAg T cell responses as identified by the IFN-γ ELISPOT assay following *ex vivo* stimulation. Figure 32 showed that the dominant epitopes are biased towards peptide pool 2.

[00239] *In vivo* cytotoxicity assay studies were performed using carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling combined with flow cytometry. Cell division at among cells of cell populations were assessed. Splenocytes were isolated from naïve mice and divided into two populations. One population, CFSE high labeled, was pulsed with relevant peptide (e.g. HBV core peptides). The other population, CFSE low labeled, was pulsed with irrelevant peptide (e.g. HCV NS3 peptides). The labeled, peptide treated cells were combined and used in adoptive transfer experiments in which flow analysis was performed. The combined populations of treated, labeled target cells were administered to two groups of mice, a control group and an immunized group. Splenocytes were isolated from each group of mice and samples were run on a flow cytometer. The amount of CFSE was measured. Typically, in such experiments, two peaks form, the first being the irrelevant peptide; the second being the immunizing peptide in the peak indicating greater CFSE.

[00240] Figure 33 demonstrate that CD8 T cell induced by vaccination can specifically eliminate target cells *in vivo*. The results demonstrate that samples of spleen and liver from naïve mice contained nearly equal amounts of cells which were in the irrelevant peptide and relevant peptide peaks while the results showed that among immunized groups, the peaks for cells derived from those pulsed with the relevant peptide were significantly less than irrelevant peptide. These data demonstrate that target cells treated with the HBV peptide

were specifically eliminated in mice immunized with the HBV vaccine but not in the non-immunized mice. Any elimination of target cells treated with the irrelevant peptide, if it occurred at all, was the same in mice immunized with the HBV vaccine and the non-immunized mice and significantly less than elimination of target cells treated with the HBV peptide.

[00241] The ability of HBV-specific CD8 T cells induced after DNA immunization to specifically eliminate target cells *in vivo* was further examined. Human CTLs that target the core antigen are important in acute clearance of HBV versus chronic infection. One week after the final immunization, 4 mice from each of the two groups, pVax or pMCore immunized, were adoptively transferred with target splenocytes that had either been pulsed with HBcAg (relevant) or HCV-NS3/4A (irrelevant) peptides. Briefly, splenocytes from naïve mice were stained with either 1μM or 1nM CFDA SE (invitrogen). The labeled splenocytes were then coated with indicated peptides (1μM) and 10^7 cells of each population intravenously injected into naïve or immunized mice. After 24 or 90 hrs, cells from the spleen and liver were isolated and analyzed by flow cytometry. The percent killing was calculated as follows: 100 - ([(% relevant peptide pulsed in infected/% irrelevant peptide pulsed in infected)/(% peptide pulsed in uninfected/% irrelevant peptide pulsed in uninfected)] x 100). By gating on CFSE labeled splenocytes to track killing, it was observed that the pMCore vaccinated mice were able to induce strong specific killing of antigen-pulsed target cells as shown in Figure 34. Average percent killing observed in the spleen was about 83% while the average in the liver was 76%, showing that vaccine-induced CTLs that migrate to and are retained in the liver are capable of killing HBV peptide pulsed target cells. This was the first study to show induction of HBcAg-specific CTL responses in the liver, by any method and specifically by systemic immunization. This data provided evidence that peripheral immunization can induce effector cells that can migrate to the liver and lyses target cells.

[00242] Figure 35 shows the data collected from the T cell Proliferation Assay using CFSE labeling. Percent proliferation of CD3+CD4+ cells and CD3+CD8+ treated with pVax vector (control) or with plasmid pMCore, which expresses HBV M-core, were compared. Briefly, the isolated splenocytes were stained with the carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) Cell Tracer Kit (Invitrogen) per the manufacturer's instructions. Stained cells were washed three times with saline and stimulated with the pMCore-specific overlapping peptides. The cells were incubated at 37°C for 96 hrs. After 48 hrs, 50% of the

culture media were removed and replaced with fresh R10. At day 4, cells were harvested and stained with CD3, CD4 and CD8-specific monoclonal antibodies (BD Pharmingen). Cells were fixed with PBS with 1% Paraformaldehyde (PFA) and acquired on a FACScalibur (Becton Dickinson). The data were analyses using FlowJo program. CFSE low and CFSE medium population was considered as proliferated cells. As shown in Figure 35, the CD3+CD8+ T cells isolated from the spleen proliferated more compared to the CD3+CD4+ T cells.

[00243] Further experiments with the T cell proliferation assay also used CFSE labeling. Particularly, isolated splenocytes were stained with the carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) Cell Tracer Kit (Invitrogen) as per the manufacturer's instructions. Stained cells were washed three times with saline and plated in a 96-well U-bottomed plate in a total volume of 200 µL of media containing HBcAg pooled peptides at a concentration of 1µg/mL. The cells were incubated at 37°C for 96 hrs. After 48 hrs, 50% of the culture media were removed and replaced with fresh R10. The difference in the cytokine production between both CD4 and CD8 T cells as discussed above was comparable to their ultimate proliferation capacity. After 4 days of stimulation with antigen specific peptides, the $CD8^+$ T cells proliferated more than 2 fold higher than the $CD4^+$ cells (Figure 36) demonstrate a clear CD8 T cell bias in the response.

[00244] Figures 37A and 37B are ELISA data showing a comparison of anti-HBV core antibody in serial dilution of sera from animals treated with pVax vector (control) or with plasmid pMCore which expresses HBV M-core. Briefly, high-binding ELISA plates (Costar, Corning, NY) were coated with 1µg/ml HBcAg protein in PBS, at 4°C for 24 h and then were washed with PBS-Tween and blocked with PBS containing 1% BSA for 2 h at room temperature. Serially diluted serum samples were added to the wells and incubated for 1 h at room temperature. After washing, bound serum Antibody was revealed by HRP-labeled goat anti-mouse IgG (Figure 37A) or IgA (Figure 37B). The peroxidase-conjugated Abs were detected using tetramethylbenzidine (Sigma-Aldrich) as the substrate, and OD at 450 nm was measured with the Multiscan ELISA Plate Reader. The antigen-specific humoral response in sera collected from immunized mice was observed.

[00245] To further explore the immune response induced in pMCore-immunized mice, antigen-specific IgG and IgA responses were analyzed by B cell ELISpot as well as in ELISA using splenocytes and sera, respectively, collected following vaccination. Splenocytes were isolated and purified as described above. For the ELISAs, high-binding ELISA plates

(Costar, Corning, NY) were coated with 1μg/ml HBcAg protein in PBS, at 4°C for 24 hrs and then were washed with 0.1% PBS-Tween then and blocked with PBS containing 1% BSA for 2 hrs at room temperature. Serially diluted serum samples were added to the wells and incubated for 1 hr at room temperature. After washing, bound serum Antibody was revealed by HRP-labeled goat anti-mouse IgA or IgG. The peroxidase-conjugated antibodies were detected using tetramethylbenzidine (Sigma-Aldrich) as the substrate, and OD values at 450 nm were measured with the Multiscan ELISA Plate Reader. A high IgG and IgA titer was observed in the sera of immunized mice when compared to control animals (Figure 38). B cell ELISpot from immunized mice (Figure 39) demonstrate HBcAg-specific IgG and IgA at approximately 200 SFU and 100 SFU per million cells, respectively. This illustrated activation of the B cell compartment by immunization with pMCore. The synthetic HBcAg plasmid effectively induced antigen-specific cellular and humoral responses after 3 immunizations.

[00246] Figure 40 demonstrates percent TNF-α and IFN-γ from CD4+ and CD8+ spleen and liver cells.

[00247] In the absence of a small animal model for HBV, HBcAg was used to transiently transfect mouse liver through hydrodynamic injection. Immunized mice livers were either transfected with pMCore or HCV NS3/4A. Immunohistochemistry staining three days post transfection showed clearance of HBcAg-transfected hepatocytes as compared to the NS3/4A-transfected ones. ALT levels in sera were measured to ensure that the clearance induced by the immunized mice did not cause any liver damage. Results in Figure 41 showed clearance induced by the immunized mice did not cause any liver damage.

[00248] Direct hydrodynamic injection was also used to transiently transfect mouse liver. Here, immunized or naive mouse livers were either transfected with pMCore or an irrelevant plasmid encoding hepatitis C antigens (HCV NS3/4A). Briefly, immunized mice were injected intravenously with 100μg of plasmid in 2mL (about 10% volume of the mouse weight) of Ringers solution within a period of 7 seconds to transiently transfect the liver. The expression or clearance of the plasmid was determined by staining the liver with anti-HA monoclonal antibodies. Immunohistochemistry staining three days post transfection (Figure 42) showed clearance of HBcAg-transfected hepatocytes as compared to the NS3/4A-transfected animal livers. CD8 T cells isolated from the pMCore hydrodynamic injected mice in Figure 43 showed a higher frequency of IFN-γ^+ CD107a$^+$, a marker of degranulation, as compared to immunized animals livers transfected with the irrelevant plasmid.

[00249] Since the clearance of pMCore-transfected hepatocytes seems to involve degranulation, it was considered that the killing may lead to liver damage. To examine if immunized mice were able to clear the transfected hepatocytes without inducing significant liver damage, an assay measuring the activity of the enzyme alanine aminotransferase (ALT) was used to indicate the presence of liver damage when elevated enzyme levels were in the sera (Figure 44). Particularly, serum alanine aminotransferase (ALT) activity was measured using an absorbance-based assay (Stanbio Laboratory) on a BioTek Synergy 2 microplate reader. Results are reported as units per liter (U/L) and represent the amount of enzyme that oxidizes one μmol/L of NADH per minute. These studies showed that the specific clearance of HBcAg-transfected hepatocytes did not increase ALT levels in transfected immunized animals beyond the normal range of 5-30U/L (Figure 44).

EXAMPLE 2

HBV Surface Proteins

1. HBsAg DNA Vaccine Design

[00250] The use of consensus immunogens in the context of variable pathogens with highly variable antigenic sequences can be effective in inducing a more direct immune response to combat native viral diversity as compared to immunization with a single native immunogen. Eight-two Hepatitis genotype A large surface antigen sequences and seventy Hepatitis genotype C large surface antigen sequences were collected from GenBank, and the genotype-specific large surface antigen and small surface antigen consensus sequences were obtained after performing multiple alignments. Clustal X (version 1.8) software was used to create multiple alignments used to generate a consensus sequence.

[00251] DNA constructs were generated encoding the major S alone or the preS1/S2 plus the major S of the HBV surface envelope protein using consensus sequences encoding for genotypes A or C, which are two of the most common genotypes in the world (Figure 52A, Figures 59A-59D). The difference in immune responses elicited by constructs encoding the full length HBs or the major S alone was examined as the amino-terminus domain of the preS1 may assist viral attachment and entry while the carboxyl terminal end of preS2 may be involved with infectivity. The major S only constructs were named small HBs (SHBs) while plasmids that contained the preS1/S2 were named large HBs (LHBs).

[00252] Consensus HBV surface proteins were designed from epitope sequences from primary isolates of HBV genotype A such that the resulting consensus protein had sequence

identities of 94.2 percent to 99.8 percent with the surface proteins of HBV genotype A primary isolates. Particularly, two versions of such a consensus protein were designed (Figure 4 and Figure 52B). One included the S protein and is also referred to as SHBs-A, in which SEQ ID NO:13 is the nucleic acid sequence and SEQ ID NO:14 is the amino acid sequence. The second included S protein, pre-S2, and pre-S1 and is also referred to as LHBs-A, in which SEQ ID NO:9 is the nucleic acid sequence and SEQ ID NO:10 is the amino acid sequence.

[00253] Consensus HBV surface proteins were also designed from epitope sequences from primary isolates of HBV genotype C such that the resulting consensus protein had sequence identities of 96.5 percent to 99.8 percent with the surface proteins of HBV genotype C primary isolates. Particularly, two versions of such a consensus protein were designed (Figure 4). One included S protein and is also referred to as SHBs-C, in which SEQ ID NO:15 is the nucleic acid sequence and SEQ ID NO:16 is the amino acid sequence. The second included S protein, pre-S2, and pre-S1 and is also referred to LHBs-C, in which SEQ ID NO:11 is the nucleic acid sequence and SEQ ID NO:12 is the amino acid sequence.

[00254] After generating consensus genotype A or C large and small surface antigen consensus sequences, codon optimization and RNA optimization was performed. For the above consensus HBV surface antigens, endoproteolytic cleavage sites were introduced into the consensus HBV surface antigens to provide for proper protein folding and better antigen processing (Figure 3). The codon usage in the consensus HBV surface antigens was modified to reflect the codon bias of human genes. Additionally, regions of very high (e.g., greater than 80 percent) or very low (e.g., less than 30 percent) GC content were avoided, as where cis-acting motifs such as internal TATA-boxes, repetitive sequences, and structured sequences. A Kozak sequence was introduced into the consensus HBV surface antigens to increase translational initiation. A highly efficient IgE leader sequence was added upstream of the start codon to enhance and increase protein expression, as previously described (Yan *et al., Vaccine* (2008) 26:5210-5215) (see Figures 3 and 52B). The optimized genes were synthesized and sequence verified.

[00255] The consensus HBV surface antigens SHBs-A, LHBs-A, SHBs-C, and LHBs-C were cloned into an expression vector to yield the constructs pSHb A (also referred to as pSHb-A), pLHb A (also referred to as pLHb-A), pSHb C (also referred to as pSHb-C), and pLHb C (also referred to as pLHb-C), respectively (Figure 4). The synthesized genotype A large and small surface antigen genes were subcloned into the expression vector pGX0001 at

BamHI and *XhoI* sites, while the synthesized genotype C large and small surface antigen genes were subcloned into pGX0001 at *EcoRI* and *NotI* sites. The generation of the HBcAg plasmid (pMCore) was described in Example 1.

2. *In vitro* expression of multiple HBsAg DNA vaccines

[00256] Rhabdomyosarcoma (RD) and human liver carcinoma (Hep G2) cell lines were transfected with pLHBs-A, pLHBs-C, pSHBs-A and pSHBs-C using TurboFect™ (Thermo Scientific) according to manufacturer's guidelines. For intracellular assay, cells were washed and fixed using the Cytofix/Cytoperm kit (BD PharMingen, San Diego, CA) according to their instructions. Following fixation, the cells were stained with sera from immunized mice or Mouse monoclonal antibody to Hepatitis B Virus Surface (Abcam, Cambridge, MA). The cells were then stained with Fluorescein isothiocyanate conjugated anti-mouse secondary (BD Biosciences, San Jose, CA). Cells were washed and fixed with 2% paraformaldehyde. Stained and fixed cells were acquired on an LSR instrument using CellQuest software (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

[00257] Following construction and optimization of the four constructs, protein expression of each plasmid was confirmed by intracellular staining. RD or Hep G2 cells were transiently transfected with individual constructs or with an empty pGX0001 vector as a control. The cells were intracellularly stained 48 hrs later using anti-HBsAg polyclonal mouse sera or a commercially acquired mouse monoclonal antibody. The expression was confirmed using flow cytometry (Figure 52C). Increased levels of HBs protein expression were observed in cells transfected with each construct over cells transfected with empty vectors (pGX0001).

EXAMPLE 3

Immunogenicity of the individual synthetic HBsAg DNA plasmids in small animal models

[00258] Following the expression studies, the ability of individual plasmids to induce antigen-specific humoral and cellular responses were evaluated *in vivo*. Six to eight week old female Balb/c mice were purchased from Jackson Laboratories. Animals were maintained in accordance with the National Institutes of Health and the University of Pennsylvania Institutional Care and Use Committee (IACUC) guidelines. Balb/c mice were given an intramuscular (IM) immunization three times, 2 weeks apart at weeks 0, 2 and 4 with 15μg of plasmids expressing either the small or large S antigen or a pGX0001 control. Each

vaccination was immediately followed by electroporation to the site of infection, as described previously (Hirao *et al., Vaccine* (2008) 26:440-448).

[00259] For immunizations, all DNA was diluted using nuclease free water (Qiagen, Valencia, CA 91355). Each animal in the immunized group received a total of three intramuscular immunizations of 15μg of the appropriate plasmid. For combined studies, each animal received a total of 45μg of DNA in both tibialis anterior (TA) muscles. Immunizations were followed by *in vivo* electroporation with the CELLECTRA® adaptive constant current electroporation device (Inovio Pharmaceuticals, Blue Bell, PA). Two 0.2 Amp constant current square-wave pulses were delivered through a triangular 3-electrode array consisting of 26-gauge solid stainless steel electrodes completely inserted into muscle. Each pulse was 52 milliseconds in length with a 1 second delay between pulses. The vaccine-induced humoral responses were examined first, because older DNA vaccines have not been consistent in generating significant antibody responses.

1. Antibody ELISA assay

[00260] An ELISA was performed to analyze antigen-specific IgG responses in sera collected from mice 7 days after each immunization using recombinant full length HBsAg protein as the target antigen. High-binding ELISA plates (Costar, Corning, NY) were coated with 1 μg/ml recombinant protein in PBS, at 4°C for 24 hrs and then were washed with 0.1% PBS-Tween and blocked with PBS containing 1% BSA for 2 hrs at room temperature. Serially diluted serum samples were added to the wells and incubated for 1 hr at room temperature. After washing, bound IgG was detected by HRP-labeled goat anti-mouse IgG. The peroxidase-conjugated antibodies were detected using tetramethylbenzidine (Sigma-Aldrich) as the substrate, and OD values at 450 nm were measured with the Multiscan ELISA Plate Reader.

[00261] Both pLHBs and pSHBs from either genotype were highly immunogenic. Further immunization boosted these responses with antibody responses showing the greatest enhancement two weeks after the third immunization (Figure 53A). In contrast, the mice immunized with the pGX0001 control only exhibited background level of IgG responses. Although, some differences in the magnitude of the IgG response were observed in each group, the progressive enhancement after each immunization was consistent within the groups.

2. IFN-γ ELISPOT

[00262] The ability of pLHBs-A, pLHBs-C, pSHBs-A and pSHBs-C constructs to induce cellular immune responses was examined by IFN-γ ELISPOT. Matching HepB consensus core, surface antigen A and surface antigen C 15-mer peptides were synthesized by Genescript (Piscataway, NJ) and resuspended in DMSO and pooled at an approximate final concentration of 1 mg/mL for each peptide. Cellular responses were measured using IFN-γ ELISpot (MabTech, Sweden) following the manufacturer's instructions. Samples were run in triplicate with an R10 (RPMI 1640 containing L-glutamine with 10% heat inactivated fetal bovine serum, and 1% Penicillin/Streptomycin) and a PMA/IM (PMA 0.1 μg/mL and ionomycin 0.5 μg/mL, Sigma Aldrich, St. Louis, MO) control.

[00263] The HBsAg-specific IFN-γ secreting cells were analyzed in response to two pools of synthetic peptides developed from consensus large HBV surface protein. The first peptide pool contained peptides spanning the preS1 and preS2 proteins while the second peptide pool was made up of peptides spanning the major S protein. All four consensus constructs were able to induce strong T cell responses and cross immune reactivity was observed within the two genotypes Figure 53B. Although, together, the preS proteins are almost similar in size to the major S protein, there was an epitope-bias in the cell-mediated immunity (CMI) responses towards the major S protein. These results indicate that the synthetic DNA immunogens encoding either the full length or only the major S portion of the HBV surface antigen exhibit immunogenicity that is diverse enough to target the major genotypes of HBV. The full length constructs showed no significant difference in the induction of IgG to HBV native protein over the shorter constructs.

3. HBV surface antigen (15-mer) peptides mapping

[00264] After confirming the magnitude of CMI, the breadth of cellular responses against genotype-specific targets was investigated. Accordingly, sets of Interferon-gamma ELISpot assays were performed against various matrix peptide pools. Two sets of peptides spanning either LBHs-A or LHBs-C protein, each containing 15 amino acid residues overlapping by 8 amino acids were synthesized by GenScript (Piscataway, NJ). In order to map the immuno-dominant epitopes, two sets of peptides were pooled into either 16 or 12 pools for the large or small HBsAg groups respectively. IFN-γ ELISpot assay was performed as described above. These different sets of pooled peptides were used in a matrix assay to map the epitopes of each construct. Responses over 50 SFU per million cells were considered to be positive.

[00265] These data illustrate that diverse epitopic responses were induced by these synthetic surface antigen plasmids in BALB/c mice. 12 out of 16 positive LHBs-A matrix pools in pLHBs-A-immunized mice, and 8 out of 16 positive LHBs-C matrix pools in pLHBs-C-immunized mice were observed. The small HBS plasmids showed higher folds of response with pSHBs-A immunized mice responding to 9 out of 12 SHBs-A matrix peptide pools, while the pSHBs-C immunized mice exhibited responses to all SHBs-C matrix pools (Figure 53C). These data indicate a high degree of broad cross-reactive cellular responses induced by these 4 HBs constructs. As a result, these genotype-specific consensus vaccines may be capable of inducing cross-reactive cellular responses against various primary surface antigen isolates that are up to 8% divergent across these genotypes.

EXAMPLE 4

Cytotoxicity Assay and Antigen-specific CTL seen in the liver and spleens of vaccinated mice

[00266] Antiviral effector CD8 T cells can induce cytotoxicity, which is a cellular activity for eliminating HBV-infected hepatocytes. The ability of the constructs to generate HBs-specific CTLs in periphery, which can also home to the liver of immunized mice, was assessed using an *in vivo* cytotoxicity assay. Splenocytes from naive mice were stained with either 1μM (high) or 1nM (low) CFDA SE (Invitrogen, Grand Island, NY, USA). The labeled splenocytes were then coated with the indicated peptides (relevant or irrelevant peptides) (1 μM) and 10^7 cells of each population were intravenously injected into naive or immunized mice. After 90 hrs, cells from the blood, spleen or liver were isolated and analyzed by flow cytometry. The percent killing was calculated as follows: 100 - ([(% relevant peptide pulsed in infected/% irrelevant peptide pulsed in infected)/(% peptide pulsed in uninfected/% irrelevant peptide pulsed in uninfected)] x 100).

[00267] Migration of HBV-specific CTLs to the liver is essential for intrahepatic elimination of infected cells. Antigen-specific elimination of adoptive transferred target splenocytes that were pulsed with HBsAg synthetic peptides was observed. The average percent killing of target cells in the spleen for mice immunized with the SHBs constructs was 39% and 48% for genotype A and C, respectively. The average target cell elimination for LHBs constructs was 35% for genotype A and 51% for genotype C. The clearance was potent but not as potent as the response observed in mice that were immunized with the core Ag plasmid alone, as described in Example 1 in which 83% and 76% target clearance was noted in the spleen and liver, respectively. The demonstration that CTL's generated against

HBs can target antigen clearance in the liver is encouraging in that it supports the combination of HBs and HBc to further expand cellular potency and limit escape. The percent elimination of target cells in the liver was 45% and 66% for SHBs-A and SHBs-C, and 60% and 74% in LHBs-A and LHBs-C groups (Figure 54). Having vaccine-specific CTLs traffic to the liver, an organ known to suppress T cell responses and induce tolerance, highlights the effectiveness of the constructs in inducing functional CMI.

EXAMPLE 5

Immunogenicity of HBsAg-HBcAg DNA cocktail

[00268] Since immunization with each synthetic HBsAg plasmid elicited broad cross-reactive immune responses, the responses were extended beyond the surface antigen to further enhance immunogenicity against diverse antigens within the virus. The HBs plasmids were combined with the plasmid encoding HBcAg (pMCore) to create an HBs-HBc DNA vaccine cocktails for immunization to determine if the cocktail will enhance the cellular response by increasing the number of immune epitopes that the T cell will recognize to eliminate since the core antigen of HBV is involved with viral clearance through the induction of HBcAg-specific CTL responses.

[00269] Five groups of mice were immunized with vaccine cocktails of HBs only antigens or HBs and HBc combined antigens. Each mouse received two shots followed by electroporation in both TAs. Mice were either immunized with cocktails containing only the surface antigens (both genotypes) or with cocktails containing both the surface antigens and the consensus core antigen plasmid (pMCore), described in Example 1 (see Table 3). The HBsAg-only immunized mice generated high IgG titer in their sera. Interestingly, similar titers were seen in the SHBs-pMCore group but were less robust in the LHBs-pMCore cocktail group (Figure 55A). The increase in antigen target may have caused some antibody inhibition.

Table 3 Immunization groups for HBs-HBc DNA cocktail mouse studies.

Mouse Groups	DNA cocktail
Group 1	pGX0001only
Group 2	pLHBs-A/pLHBs-C
Group 3	pSHBs-A/pSHBs-C
Group 4	pLHBs-A/pLHBs-C/pMCore

Group 5	pSHBs-A/pSHBs-C/pMCore

[00270] To further explore the immune response elicited by the HBs-HBc immunized mice, the production of IFN-γ from each immunized group was evaluated. The average SFU per million splenocytes for groups with no pMCore were about 689 and 666 for genotype A and 1160 and 1312 against genotype C peptides, respectively. Moreover, the addition of pMCore on average increased the total SFU per million splenocytes by 400 spots against genotype C and doubled the response when the splenocytes were stimulated with genotype A peptides (Figure 55B).

[00271] CD8 T cells are known to be the main effector cells responsible for HBV clearance. It is important to determine which T cell type is responsible for the observed IFN-γ production and to examine if these T cells can induce multiple antiviral functions in response to antigenic re-encounter by releasing other antiviral cytokines, such as TNF-α and IL-2 and degranulation markers, such as CD107a. The ability of CD8 T cells from immunized mice to induce these cytokines and markers were assessed after ex vivo stimulation with the appropriate peptides. Multi-color flow analysis revealed that a significant percentage of CD8 T cells from each group were polyfunctional (i.e., produced multiple cytokines) activated CD8 T cells within all groups (Figures 56A - F). Also observed was the antigen specific induction of the lymphocyte degranulation marker, CD107a in these CD8 T cells. CD8 T cells from HBs-HBc cocktail groups showed higher poly-functional activity when stimulated with the surface antigen peptides, confirming a synergistic effect in CD8 T cell antiviral cytokines like IFN-γ TNF-α and IL-2, and degranulation marker, CD107a. The induction of CTLs through the clearance of transferred antigen pulsed cells was also observed. While there was reduction in antibody responses to HBs for cocktails including the LHBs constructs, SHBs-pMCore vaccine cocktails maintained the humoral responses detected in animals immunized only with SHBs.

EXAMPLE 6

Immunization of Rhesus macaques with cocktail induces strong cellular HBc- and HBs-specific and humoral HBs-specific immune responses

[00272] DNA vaccine potency of prior DNA constructs have been consistently weak in larger animals and humans. The strong antibody and T cell responses incited by the combination of pLHBs or pSHBs and pMCore in mice lead to the evaluation of the immunogenicity of these constructs in a non-human primate model (NHP), which more

closely mimics the human immune response. This is a hurdle for vaccine immunogenicity as the larger size and outbreed MHC population of NHP typically results in reduced or non-existent immunogenicity compared with mouse models. For these studies, IL-12, a Th1-driving cytokine, was added to be analyzed. IL-12 has been shown to drive CMI by aiding the priming and expansion of CD8 T cells, when added to an immunization as an adjuvant to enhance the immune response against different antigens from various pathogens. This adjuvant increases T cell responses of DNA vaccine in mice and in macaques and humans.

1. Vaccination

[00273] Fifteen Indian rhesus macaques were housed at Bioqual (Rockville, MD) according to the standards of the American Association for Accreditation of Laboratory Animal Care. The rhesus macaques monkeys were placed in groups, in which each group had 5 monkeys. *See* Table 4.

Table 4 Non-human primate immunization groups

Monkey Groups	DNA cocktail
Group A	pSHBs-A/pSHBs-C/pMCore
Group B	pLHBs-A/pLHBs-C/pMCore
Group C	pLHBs-A/pLHBs-C/pMCore + IL-12

[00274] One group was administered via intramuscular (IM) delivery (also known as *in vivo* electroporation) a vaccine including 1.0 mg of each of the constructs pMCore, pSHb A, and pSHb C; this group is referred to as the "small" group. A second group was administered via IM delivery a vaccine including 1.0 mg of each of the constructs pMCore, pLHb A, and pLHb C; this group is referred to as the "long" group. A third group was administered via IM delivery a vaccine including 1.0 mg of each of the constructs pMCore, pLHb A, and pLHb C, and 0.4 mg of prhIL-12 (plasmid expressed optimized rhesus IL-12); this group is referred to as the "long + IL-12" or "pLHBs+12" group. The construct prhIL-12 encodes the Rhesus macaque IL-12 protein. DNA was delivered to a single site in the quadriceps followed by *in vivo* EP with the constant current CELLECTRA® device (Inovio Pharmaceuticals, Blue Bell, PA) with 3 pulses at 0.5 A constant current, a 52 ms pulse length and 1 s rest between pulses. Animals were vaccinated at weeks 0, 4, 12 and 30.

2. Sample collection

[00275] Samples were harvested from the monkeys prior to immunization at week 0 and also after each immunization (i.e., after weeks 0, 4, and 12). Animals were bled 2 weeks following each immunization. Blood (20 mL at each time point) was collected in EDTA tubes and PBMCs were isolated using standard Ficoll-Hypaque procedure with Accuspin tubes (Sigma-Aldrich). An addition 5 mL blood was collected into clot tubes and sera aliquotted for analysis.

[00276] High titer antibody responses against HBsAg were observed after two immunizations with pSHBs/pMCore but not with pLHBs/pMCore. This response was comparable to the mouse antibody data of Figure 55A. The response against pLHBs/pMCore was boosted after the third immunization. The IL-12 adjuvant enhanced the antibody titer of the pLHBs/pMCore cocktail immunization as early as after just two immunizations (Figure 57A).

[00277] The cellular immune responses from PBMCs show little to no HBs or HBc-specific responses following the first immunization. However, responses were observed after the second immunization and boosted after each subsequent immunization. Robust responses of over 2000 SFU per million PBMCs were observed in all groups after the third immunization and were largely targeted against the core antigen. The addition of the IL-12 adjuvant enhanced the magnitude of the T-cell response 1000 SFU per million PBMCs compared with the pLHBs/pMCore cocktail alone (Figure 57B). To further define the breadth of the cellular response, matrix pools of the consensus core peptides were created to determine the number of possible epitopes. The T cell responses spanned across the multiple peptide pools with an average response to 12 peptide epitopes (Figure 57C). These responses are consistent with those previously as seen in the small animal models. These results suggest there cocktails are capable of inducing cellular responses with both robust magnitude and breadth and that the magnitude of these responses may be further enhanced by the addition of IL-12.

[00278] Figures 45-48 show the cellular immune responses induced by vaccines administered to the small, long, and long+IL-12 groups. An enzyme-linked immunosorbent spot (ELISPOT) assay was utilized to determine the cellular immune responses as detailed above in Example 1. As depicted in Figures 45-48, the T-cell response was boosted with each vaccination. However, the HBV core antigen was more immunogenic than HBV surface antigen A and HBV surface antigen C. The data also demonstrates that longer

consensus HBV surface antigen (i.e., includes S protein, pre-S2, and pre-S1) is as immunogenic as the smaller consensus HBV surface antigen (i.e., includes S protein).

[00279] Similar to Figures 45-48, Figure 49 shows the cellular immune responses induced by vaccines administered to the small, long, and long+IL-12 groups as measured by the ELISPOT assay, however, different pools of peptides covering the core antigen, surface antigen A, and surface antigen C were examined. Again, the T-cell response was boosted with each vaccination, the addition of IL-12 enhanced the cellular immune response, and the long and short consensus HBV surface antigens were similarly immunogenic.

[00280] Figure 50 shows ELISA data comparing anti-HBV antibody responses for the small, long, and long+IL-12 groups. ELISA's were performed as detailed above in Example 1. Antigen-specific humoral responses in sera collected from the immunized monkeys were observed. Particularly, the vaccines including the long consensus surface antigen (i.e., S protein, pre-S2, and pre-S1 has a better antibody response, which can be due to the extra epitopes present in the vaccine.

[00281] Figure 51 shows ELISA data comparing anti-HBV antibody responses for the long+IL-12 group prior to immunization (prevac) and after immunization at week 0, week 4, and week 12 (i.e., post 1^{st}, post 2^{nd}, and post 3^{rd}, respectively). ELISAs were performed as described above in Example 1. The data in Figure 51 demonstrated that 2 immunizations or doses are required for measurable antibody production. Furthermore, a significant boost in the anti-HBV antibody response was observed after the third immunization or dose.

EXAMPLE 7

[00282] Similar to the experiments with mice in Example 5, splenocytes, *i.e.*, CD8 and CD4 T-cells, were isolated from the immunized Rhesus monkeys of Example 6 to examine the levels of INF-γ and TNF-α secretion in response to administration of the combination of consensus core and consensus surface antigens. The CD8 and CD4 T-cells were studied via polyfunctional and phenotypic flow analysis to examine the secretion of tumor necrosis factor, interferon gamma, and CD107a from these CD8 and CD4 T-cells to determine if the CD8 and CD4 T cells of the immunized monkeys from Example 6 have similar characteristics as the mouse CD8 and CD4 T cells studied in Example 1.

[00283] To further define the function of the cellular immune response, intracellular staining for antiviral cytokines was performed following stimulation with core and surface antigens from A and C genotypes. Intracellular staining with antibodies to antiviral cytokines revealed that antigen-specific production of antiviral cytokines (IFN-γ, TNF-α and IL-2) was

induced in both CD4 and CD8 T cells from immunized monkeys. Figure 57D shows that following three immunization, both HBV-specific CD4 and CD8 T cells responses were observed. Similar to the results seen with the IFN-γ ELISpot, responses against the core antigen were dominant. The addition of IL-12 enhanced the overall magnitude of the response however, as expected this effect was more pronounced for the CD8 T cells. The induction of both CD4 and CD8 responses averaging 1% suggests the induction of a broad immune response consisting of both Th1 and Th2 immunity and is strongly encouraging for the advancement of this strategy in human trials.

[00284] The antibody and T cell responses to both HBs and HBc antigens were similar to the results generated with mice in Example 5. These responses were even more impressive when IL-12, a Th1 polarizing cytokine adjuvant, was added to the pLHBs-pMCore cocktail. It appears that the pLHBs-pMCore + IL-12 formulation was highly effective as a preferred combination. It is noteworthy that no vaccine formulation resulted in the elevation of parameters associated with liver damage.

EXAMPLE 8

Analysis of Liver Function in Non Human Primates Post Vaccination Using Liver Blood Chemistry Panels

[00285] As clinical studies of patients actively infected with HBV have suggested the possibility that a T cell response may be linked to liver pathology during disease, preliminary analysis of the gross safety of these multivalent vaccines in regards to liver function was performed. In order to examine liver function, serum levels of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin were analyzed in the vaccinated animals prior to immunization, after the third vaccination, and just prior to the fourth vaccination and after the fourth vaccination. Blood Chemistry panels were run by IDEXX Laboratories and included alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin. Results of this analysis are shown in Figure 58. A lack of elevation of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and total bilirubin in liver function tests prior to, during and after completion of the immunization period suggested that the induction of HBV-specific immune responses was not inducing significant damage against the liver of vaccinated animals.

[00286] Taken together, the data supports that administration of this synthetic DNA vaccine cocktail encoding designed HBV antigens in combination with specific electroporation drives

robust and diverse cellular and humoral responses in both mice and NHP while avoiding gross toxicity.

[00287] It is understood that the foregoing detailed description and accompanying examples are merely illustrative and are not to be taken as limitations upon the scope of the invention, which is defined solely by the appended claims and their equivalents.

[00288] Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to the chemical structures, substituents, derivatives, intermediates, syntheses, compositions, formulations, or methods of use of the invention, can be made without departing from the spirit and scope thereof.

The invention claimed is:

1. An immunogenic composition comprising:

(a) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO:10 and a protein that is 95% identical to the full length amino acid sequence of SEQ ID NO:10; or

(b) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO: 12 and a protein that is 95% identical to the full length amino acid sequence of SEQ ID NO:12.

2. The immunogenic composition of claim **1**, further comprising:

(c) a nucleic acid molecule encoding SEQ ID NO:2.

3. The immunogenic composition of claim **1**, comprising:

(a) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO:10 and a protein that is 95% identical to the full length amino acid sequence of SEQ ID NO:10;

(b) a nucleic acid molecule encoding one or more proteins selected from the group consisting of: SEQ ID NO: 12 and a protein that is 95% identical to the full length amino acid sequence of SEQ ID NO:12; and

(c) a nucleic acid molecule encoding SEQ ID NO:2.

4. The immunogenic composition of claim **3**, wherein the nucleic acid molecules comprise one or more nucleotide sequences selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:9, and SEQ ID NO:11.

5. The immunogenic composition of claim **1**, wherein the nucleic acid molecules are plasmids.

6. The immunogenic composition of claim **1**, wherein the nucleic acid molecules are incorporated into viral particles.

7. The immunogenic composition of claim 1 further comprising an adjuvant molecule.

8. The immunogenic composition of claim **7**, wherein the adjuvant is IL-12, IL-15, IL-28, or RANTES.