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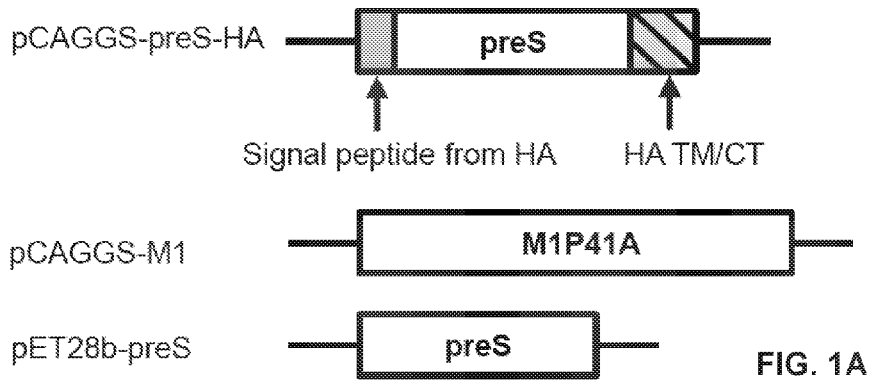


FIG. 1A

(57) Abstract: As disclosed herein, the preS antigen on infectious hepatitis B virus (HBV) particles can provide B and T ceil epitopes that promote the humoral and cellular responses and enhance the seroprotection rate by overcoming non-responsiveness to the S antigen-only vaccines. Therefore, compositions and methods are disclosed using the preS antigen to develop vaccines and immune therapies for treating or preventing hepatitis B infection, in particular, virus-like particles (VLPs) are disclosed that contain the preS antigen on its surface. These VLPs can be used alone or in combination with vaccines containing the hepatitis B surface antigen (HBsAg) to vaccinate subjects against HBV as well as to activate T ceils for adoptive T cell therapy to eradicate HBV infected hapatocytes.

WO 2018/080889 A1

A VIRUS LIKE PARTICLE OF HEPATITIS B VIRUS PRE-S PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Application No. 62/414,899, filed October 31, 2016, which is hereby incorporated herein by reference in its entirety.

BACKGROUND

Despite great progress in antiviral treatments, hepatitis B virus (HBV) infection is still a major global public health problem. Approximately 2 billion people have been infected worldwide during their lifetime, and more than 350 million are chronic carriers of the virus (Liaw YF, et al. Lancet 2009 373:582-592). HBV infection may cause acute and chronic hepatitis, which leads to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (Chu CM. J Gastroenterol Hepatol 2000 15 Suppl:E25-30). Current HBV vaccines on the market which protect most people against HBV infection contain only the S antigen. However, almost 5-10% people vaccinated with the available vaccines fail to mount an adequate antibody response to offer protection (Kubba AK, et al. Commun Dis Public Health 2003 6:106-112).

SUMMARY

As disclosed herein, the HBV preS protein, or an antigenic fragment thereof, can provide B and T cell epitopes that promote the humoral and cellular responses and enhance the seroprotection rate by overcoming non-responsiveness to the S antigen-only vaccines. Therefore, compositions and methods are disclosed using a preS antigen to develop vaccines and immune therapies for treating or preventing hepatitis B infection.

In some embodiments, a preS antigen is incorporated into a virus-like particle (VLP) that can be used, for example, as a vaccine or to active T cells for adoptive cell transfer. In these embodiments, the preS antigen can be incorporated into a fusion protein that will incorporate into a VLP. For example, a fusion protein is disclosed that comprises a hepatitis B preS antigen fused at the N-terminus to a transmembrane domain and optional cytoplasmic tail of a viral envelope protein. Viral envelope proteins that contain transmembrane domains suitable for VLP formation include influenza virus hemagglutinin (HA) protein, a type I transmembrane protein. The hepatitis B preS antigen may also be fused with other type I transmembrane glycoproteins, such as glycoproteins from arenaviruses, bunyaviruses,

coronaviruses, filoviruses, paramyxoviruses, retroviruses, togaviruses, and others (Liu J, et al. VIROLOGICA SINICA 2016 31 (4): 279–287).

In some embodiments, the fusion protein further contains a signal peptide at the N-terminus. As an example, the signal peptide can be the signal peptide from HA. The signal peptide may also be derived from other type I transmembrane glycoproteins, such as glycoproteins from arenaviruses, bunyaviruses, coronaviruses, filoviruses, paramyxoviruses, retroviruses, togaviruses, and others (Liu J, et al. VIROLOGICA SINICA 2016 31 (4): 279–287).

The fusion protein can be formed into a VLP by co-expressing it with a viral matrix protein, such as influenza virus matrix protein 1 (M1). The fusion protein can also be formed into a VLP by co-expressing it with other viral proteins, including matrix protein, nucleocapsid protein, and other proteins from arenaviruses, bunyaviruses, coronaviruses, filoviruses, paramyxoviruses, retroviruses, togaviruses, and others (Liu J, et al. VIROLOGICA SINICA 2016 31 (4): 279–287). Therefore, also disclosed are VLPs comprising the disclosed fusion protein and an influenza virus M1 protein. These VLPs can be produced, for example, by introducing expression vectors into mammalian cells (e.g. Chinese hamster ovary (CHO), human cell line 293, and Vero cells (money cells)) as transient or stable expression, or coinfecting insect cells with one or more recombinant baculoviruses expressing the M1 protein and the disclosed fusion protein, culturing the cells under appropriate conditions. VLPs can also be produced by the same methods when other viral proteins are employed in constructing the fusion protein and coexpression. The VLPs can then be purified from cell culture supernatants.

Also disclosed are vaccines comprising an effective amount of the disclosed VLPs in a pharmaceutically acceptable excipient. In some cases, the vaccine further comprises an adjuvant. For example, the adjuvant can be selected from the group consisting of AS04 (alum plus monophosphoryl lipid A), bacterial cell wall components, MF59 (mineral oil based adjuvant), and a molecular adjuvant incorporated VLP in a membrane-anchored form.

Also disclosed is an isolated polynucleotide comprising a nucleic acid sequence encoding the disclosed fusion protein can be constructed following the amino acid codons. In some embodiments, the nucleic acid sequence encoding the fusion protein is operably linked to an expression control sequence. Therefore, also disclosed is an expression vector comprising the disclosed nucleic acid sequence. Also disclosed herein is a cell comprising the disclosed vector. For example, the cell can be a bacterium, insect cell, mammalian cell or yeast cell.

Also disclosed is a method of vaccinating a subject for hepatitis B comprising administering a vaccine disclosed herein to a subject in need thereof by intranasal, intramuscular, subcutaneous, transdermal, or sublingual administration. The method can further involve administering to the subject a vaccine comprising the hepatitis B surface antigen (HBsAg).

Also disclosed is a method for activating CD8+ T cells for adoptive cell transfer (ACT), comprising co-culturing CD8+ T cells and dendritic cells with a VLPs disclosed herein.

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

DESCRIPTION OF DRAWINGS

Figures 1A to 1D show construction and expression of preS VLP. Figure 1A is a schematic representation of pCAGGS-preS-HA and pCAGGS-M1 used for preS VLP production, and pET28b-preS used for the expression of preS antigen. Figure 1B shows results of qPCR analyses used to measure the transcription of preS-HA and M1. Figures 1C and 1D are immunofluorescent images of preS-HA expressing cells. 293T cells were transfected with mock control, pCAGGS-M1, pCAGGS-preS-HA, or both plasmids. Cells either were (Fig. 1D) or were not (Fig. 1C) permeabilized with Triton X-100 before staining. The nuclei were stained with DAPI, and the preS antigen was stained with an anti-preS sera, detected with Alexa Fluor® 488-Conjugated goat anti-rabbit secondary antibody.

Figures 2A to 2E shows characterization of preS VLP. Figure 2A shows Western blot analysis of preS-HA in lysates of plasmid transfected cells and cognate supernatant. Figure 2B shows SDS-PAGE analysis of samples from fractions from sucrose gradient centrifugation. The major protein component is in the 40% sucrose fraction. Figure 2C shows Western blot analysis with anti-preS sera. Lane 1 was for recombinant preS protein expressed in *E. coli*. Lanes 2 and 3 showed the presence of the preS-HA antigen in fractions from sucrose gradient centrifugation. Figure 2D is an electron micrograph showing preS virus-like particles (preS VLP). Magnification, 11,000 ×. Figure 2E LC-MS/MS identification of M1 and preS-HA in the sample from 40% sucrose fraction.

Figures 3A to 3D show preS VLP elicits superior HBV-specific humoral immune responses compared to recombinant preS vaccination. Figures 3A to 3C show serum anti-preS titers determined by ELISA. The plates were coated with

5 μg/mL recombinant preS. The immunization condition is labeled next to chart codes. The sera were diluted by 100 fold in all the assays. Figure 3D shows neutralization of HBV infectivity in HepG2/hNTCP cells by mouse anti-preS VLP sera or hepatitis B immunoglobulin (HBIG) (0.144 mg/mL). The mouse anti-preS VLP sera were diluted by 10 folds. HBeAg values at one week post infection were measured using an ELISA kit. The data are represented as mean ± SEM.

Figures 4A to 4E show preS VLP induces stronger T cell responses than recombinant preS vaccination. Balb/c mice were immunized intramuscularly with preS VLP (n=6), recombinant preS protein (n=6), or PBS (n=6). 30 days post immunization, splenocytes were isolated and analyzed for CD8 (Fig. 4A, 4C), CD4 (Fig. 4B, 4D), and IFN-γ (Fig. 4C, 4D) expression by flow cytometry (Figs. 4A-4D) or ELISPOT assays (Fig. 4E).

Figures 5A to 5F show vaccination with preS VLP offers protection against hydrodynamic HBV challenge. Balb/c mice were immunized intramuscularly with preS VLP (n=6), recombinant preS protein (n=6), or PBS (n=6). On day 70, HBV replication was induced by hydrodynamic injection of pT-HBV1.3 plasmid via tail vein (10 μg per mouse). Figure 5A shows liver-associated HBV RNA copies measured by qPCR. Figure 5B shows immunohistochemistry analyses of liver tissues. Magnification, 100×. Figures 5C to 5E show serum analysis for HBsAg (Fig. 5C), HBeAg (Fig. 5D) and anti-preS antibody titers (Fig. 5E) completed by ELISA on days 0, 2, 4, and 7 post-challenge. Figure 5F shows the serum alanine aminotransferase (ALT) activity that was determined with a Hitachi 7600 Automatic Biochemistry Analyzer. All values are presented as the average from each group, and error bars indicate ± SEM.

Figures 6A to 6F show preS VLP-mediated protection correlates with T cell recall response. Balb/c mice were immunized intramuscularly with preS VLP (n=6), recombinant preS protein (n=6), or PBS (n=6). On day 70, HBV replication was induced by hydrodynamic injection of pT-HBV1.3 plasmid (10 μg). 7 days postchallenge, splenocytes (Figs. 6A-6E) and intrahepatic lymphocytes (Fig. 6F) were isolated and analyzed for CD8, CD4, and IFN-γ expression by flow cytometry (Figs. 6A-6D) or ELISPOT assays (Figs. 6E-6F).

Figures 7A to 7J show immunization with preS VLP induces robust anti-preS antibodies and T cell responses in HBV transgenic mice. HBV transgenic mice were immunized intramuscularly with preS VLP (n=6) or PBS (n=6). Figure 7A is a time schedule for preS VLP as a vaccine to treat HBV transgenic mice. Figures 7B to 7D show serum anti-preS titers determined by ELISA. The plates were coated with 1 μg/mL purified recombinant preS. The immunization condition is labeled next to chart

color codes. The sera were diluted by 100 folds in all the assays. CD8+ T cells (Fig. 7E) or CD4+ T cells (Fig. 7F) were gated, and IFN-γ-producing cells are presented as the percent average from each group. Figure 7G shows IFN-γ expression by ELISPOT assays in splenocytes isolated on day 70. ELISPOT experiments were performed in triplicate wells per condition. Figure 7H shows representative images of ELISPOT from each group. All values are presented as the average from each group, and error bars indicate ± SEM. Figure 7I shows the Serum alanine aminotransferase (ALT) activity that was measured using a Hitachi 7600 Automatic Biochemistry Analyzer.. Figure 7J shows liver tissue sections from each group that were stained with hematoxylin and eosin. Magnification, 100×.

DETAILED DESCRIPTION

Definitions

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a peptide" includes a plurality of such peptides, reference to "the peptide" is a reference to one or more peptides and equivalents thereof known to those skilled in the art, and so forth.

In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where the event or circumstance occurs and instances where it does not. For example, the phrase "optionally a signal peptide" means that the signal peptide may or may not be included.

The term "universal influenza A vaccine" refers to vaccine capable of providing cross-protection against at least two, including three, four, five or more, subtypes of influenza A.

The term "individual," "host," "subject," and "patient" are used interchangeably to refer to any individual who is the target of administration, treatment, or vaccination. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human or veterinary patient.

The term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

The term "carrier" means a compound, composition, substance, or structure that, when in combination with a compound or composition, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or composition for its intended use or purpose. For example, a carrier can be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

The terms "peptide," "protein," and "polypeptide" are used interchangeably to refer to a natural or synthetic molecule comprising two or more amino acids linked by the carboxyl group of one amino acid to the alpha amino group of another.

The term "protein domain" refers to a portion of a protein, portions of a protein, or an entire protein showing structural integrity; this determination may be based on amino acid composition of a portion of a protein, portions of a protein, or the entire protein.

The term "nucleic acid" refers to a natural or synthetic molecule comprising a single nucleotide or two or more nucleotides linked by a phosphate group at the 3' position of one nucleotide to the 5' end of another nucleotide. The nucleic acid is not limited by length, and thus the nucleic acid can include deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

The term "variant" refers to an amino acid sequence having conservative amino acid substitutions, non-conservative amino acid substitutions (i.e. a degenerate variant), or a peptide having 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%$, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the recited sequence.

The term "percent (%)sequence identity" or "homology" is defined as the percentage of nucleotides or amino acids in a candidate sequence that are identical with the nucleotides or amino acids in a reference nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods. For purposes herein, the % sequence identity of a given nucleotides or amino acids sequence C to, with, or against a given nucleic acid sequence D (which

can alternatively be phrased as a given sequence C that has or comprises a certain % sequence identity to, with, or against a given sequence D) is calculated as follows:

100 times the fraction W/Z,

where W is the number of nucleotides or amino acids scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides or amino acids in D. It will be appreciated that where the length of sequence C is not equal to the length of sequence D, the % sequence identity of C to D will not equal the % sequence identity of D to C.

A "fusion protein" refers to a polypeptide formed by the joining of two or more polypeptides through a peptide bond formed between the amino terminus of one polypeptide and the carboxyl terminus of another polypeptide. The fusion protein can be formed by the chemical coupling of the constituent polypeptides or it can be expressed as a single polypeptide from nucleic acid sequence encoding the single contiguous fusion protein. A single chain fusion protein is a fusion protein having a single contiguous polypeptide backbone. Fusion proteins can be prepared using conventional techniques in molecular biology to join the two genes in frame into a single nucleic acid, and then expressing the nucleic acid in an appropriate host cell under conditions in which the fusion protein is produced.

A "spacer" as used herein refers to a peptide that joins the proteins of a fusion protein. Generally a spacer has no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of a spacer may be selected to influence some property of the molecule, such as the folding, net charge, or hydrophobicity of the molecule.

Hepatitis B preS constructs

Disclosed herein is a fusion protein comprising a heptatis B preS epitope fused to a membrane anchor domain, such as a transmembrane domain and optional cytoplasmic domain of a viral envelope protein, for use in producing virus-like particles.

For example, the heptatis B preS epitope sequences can be derived from human. In some embodiments, the hepatitis B preS antigen has the amino acid sequence MGT NLSVPNPLGF FPDHQLDPAF GANSNNPDWD FNPIKDHWPA ANQVGVGAFG PGLTPPHGGI LGWSPQAQGI LTTVSTIPPP ASTNRQSGRQ PTPISPPLRD SHPQAMQWNS TAFHQALQDP RVRGLYLPAG GSSSGTVNPA PNIASHISSI SARTGDPVTN (SEQ ID NO:1), or a conservative variant thereof having at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,

98%, 98%, 99%, or 100% sequence identity to SEQ ID NO:1 (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitutions).

The hepatitis B virus preS antigen from other genotypes or subtypes can also be used to produce VLPs by the same methods.

In some embodiments, the influenza virus M1 protein has the amino acid sequence MSLLTEVETY VLSIIPSGPL KAEIAQRLEG VFAGKNTDLE ALMEWLKTRP ILSPLTKGIL GFVFTLTVPS ERGLQRRRFV QNALNGNGDP NNMDRAVKLY KKLKREITFH GAKEVSLSYS TGALASCMGL IYNRMGTVTT EAAFGLVCAT CEQIADSQHR SHRQMATTTN PLIRHENRMV LASTTAKAME QMAGSSEQAA EAMEVASQTR QMVHAMRTIG THPSSSAGLK DDLLENLQAY QKRMGVQIQR FK (SEQ ID NO:3), or a conservative variant thereof having at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98%, 99%, or 100% sequence identity to SEQ ID NO:3 (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitutions).

The transmembrane-cytoplasmic domain from hemagglutinin can have the amino acid sequence KLESVGVHQI LAIYSTVASS LVLLVSLGAI SFWMCSNGSL QCRICI (SEQ ID NO:15), or a conservative variant thereof having at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98%, 99%, or 100% sequence identity to SEQ ID NO:15.

The fusion protein may further comprise a signal peptide at the N-terminus to facilitate secretion. For example, the fusion protein may contain a hemagglutinin (HA) signal peptide. The signal peptide from hemagglutinin can have the amino acid sequence MEAKLFVLFC AFTALKA (SEQ ID NO:16), or a conservative variant thereof having at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98%, 99%, or 100% sequence identity to SEQ ID NO:16. Other signal peptides are known and include those listed in Table 1 below.

Table 1. Signal Peptides

Accession Number	Signal Sequence	SEQ ID NO
Q6DQ15	VLLLAIVSLVKS	SEQ ID NO:17
P16060	MERIVIALAIISVVKG	SEQ ID NO:18
P03446	MEKFIILSTVLAASFAY	SEQ ID NO:19
Q6LEJ4	MNTQILVFIACVLIEAKG	SEQ ID NO:20
P09344	MNTQILILTLVAAIHTNA	SEQ ID NO:21
P16561	MARLPILLLLISLVYS	SEQ ID NO:22
P03459	MNTQILVFALVAVIPTNA	SEQ ID NO:23
P03436	MKTIIALSYIFCLALG	SEQ ID NO:24
P03445	MKKVLLFAAIIICIRA	SEQ ID NO:25
Q82559	MKTTIILILLTHWVYS	SEQ ID NO:26

P16996	MKTTIVLILLTHWVYS	SEQ ID NO:27
Q67371	MKAIIVLLMVVTSNA	SEQ ID NO:28
Q80A30	SLVKS	SEQ ID NO:29
P04659	MEKTLLFAAIFLCVKA	SEQ ID NO:30
Q1PUD9	MKTIIALSYIFCLVLG	SEQ ID NO:31
P26138	MKTIIVLSCFFCLAFS	SEQ ID NO:32
P13102	MDIRPIIISLLISTCVQA	SEQ ID NO:33
P10448	LMVVTSNA	SEQ ID NO:34
P12443	IVLLMVVTSNA	SEQ ID NO:35
Q6DQ18	AIVSLVKS	SEQ ID NO:36
P26097	MNTQILILATSAFLCVRA	SEQ ID NO:37
P03443	MLSITILFLLIAEGSS	SEQ ID NO:38
Q9WCE3	MEAKLLVLFCTFAALKA	SEQ ID NO:39
P08714	MTRLPILLLLISLVYA	SEQ ID NO:40
P19696	MLSIVILFLLIAENSS	SEQ ID NO:41
P16994	MKTTIILILLIHWVHS	SEQ ID NO:42
Q03909	MLSLIMRTVIALSYIFCLAFG	SEQ ID NO:43
Q6DQ19	MEKIVLLLAIVSLVKS	SEQ ID NO:44
P17002	MKTTTILILLTHWVHS	SEQ ID NO:45
P18875	MKAKLLVLLCALSATDA	SEQ ID NO:46
P03451	MAIIYLILLFTAVRG	SEQ ID NO:47
P26100	MNTQILILAISAFLCVRA	SEQ ID NO:48
P11132	MEEIVLLFAIVSLARS	SEQ ID NO:49
O89746	MEKIVLLLATVSLVKS	SEQ ID NO:50
P03454	MKAKLLVLLYAFVATDA	SEQ ID NO:51
Q0A3Y1	MDIRAIVISLLISTCVQA	SEQ ID NO:52
P87506	MERIVIALAIINIVKG	SEQ ID NO:53
P26562	MEAKLFVLFCTFTVLKA	SEQ ID NO:54
P12583	MKTIIALSYIFCLAFS	SEQ ID NO:55
P12590	MKAKLLVLLCAFTATDA	SEQ ID NO:56
P33807	MTRLSILLLLISLVYS	SEQ ID NO:57
Q9WFX3	MEARLLVLLCAFAATNA	SEQ ID NO:58
P03456	MEKFIAIATLASTNAY	SEQ ID NO:59
Q9WCD8	MKAILLVLLCAFAATNA	SEQ ID NO:60
Q30NQ1	MKTIIALSYIFCLAFA	SEQ ID NO:61
Q2VND2	MKTIIALSYIFCQVFA	SEQ ID NO:62
P13101	MDIQAVALLILTSTCVQA	SEQ ID NO:63
Q00716	MKQLSIVILLLSIVYT	SEQ ID NO:64
Q67282	MTITFLILLFTVVKG	SEQ ID NO:65
Q38SQ8	MKTIIALSYIFCLVFA	SEQ ID NO:66
P13103	MALNVIATLTLISVCVHA	SEQ ID NO:67
P07976	MERTVIALAIISVVKG	SEQ ID NO:68

P12440	MVVTSNA	SEQ ID NO:69
Q6DQ20	MKKIVLLLAIVSLVKS	SEQ ID NO:70
P03442	MKTVIALSYILCLTFG	SEQ ID NO:71
Q82509	MERIVLFLAIVSLVKS	SEQ ID NO:72
P26135	MKTIIVLSYFFCLALS	SEQ ID NO:73
P12439	MYKVVVIIALLGAVKGL	SEQ ID NO:74
Q6DQ22	KIVLLLAIVSLVKS	SEQ ID NO:75
P26094	MNTQILILATSAFFYVRA	SEQ ID NO:76
P03447	MERVVLLLAMISLVKS	SEQ ID NO:77
P26141	MKTLIALSYIFCLVLG	SEQ ID NO:78
P36346	MNTQILVFALVAVIHTNA	SEQ ID NO:79
P03448	MIAIIVVAILATAGRS	SEQ ID NO:80
P11135	MEKIVLLFAIVSLVRS	SEQ ID NO:81
O56140	MEKTVLLLATVSLVKS	SEQ ID NO:82
P09345	MERIVLLLAIVSLVKS	SEQ ID NO:83
P19694	MLSVVILFLLVAENSS	SEQ ID NO:84
P04660	MKKILLFTVIFLYAKA	SEQ ID NO:85
P04662	MEKLLLFATIILCVKA	SEQ ID NO:86
P19698	MLSIVVLLLLIAESSS	SEQ ID NO:87
Q9WCE8	MEAKLFVLFCAFTTLEA	SEQ ID NO:88
P03449	MKTIIALSHIFCLVLG	SEQ ID NO:89
P19697	MLSIVVLLLLMAEGSS	SEQ ID NO:90
P26136	MIALILVALALSHTAYS	SEQ ID NO:91
P26140	MKAILLVLLYTFTAANA	SEQ ID NO:92
P03452	MKANLLVLLCALAAADA	SEQ ID NO:93
P03455	MKAILLVLLCTFAATNA	SEQ ID NO:94
P28730	MKAKLLILFCAFTATDA	SEQ ID NO:95
Q6J8F6	MEKIVLLFAIVSLVKS	SEQ ID NO:96
P03457	METKAIIAALLMVTAANA	SEQ ID NO:97
P19700	MLSITILFLLIAEVSS	SEQ ID NO:98
P03440	MKTIIALSYIFCQVLA	SEQ ID NO:99
P28731	MKAKLLVLFCAFTATDA	SEQ ID NO:100
P03461	VTSNA	SEQ ID NO:101
P12581	MYKVVVIIALLGAVRGL	SEQ ID NO:102
P26137	LVALALSQTAYS	SEQ ID NO:103
P12441	IIVLLMVVTSNA	SEQ ID NO:104
P03444	MYKIVLVLTLFGAVNGL	SEQ ID NO:105
P03458	MNTQILVFIACVLIKAKG	SEQ ID NO:106
O11283	MKTIIALSYILCLVFA	SEQ ID NO:107
Q9QAQ9	MFLLPRFVLVSCIIGSLG	SEQ ID NO:108
P31615	MCIAMAPRTLLLLIXCQLVF	SEQ ID NO:109
P15776	MFLLLRFVLVSCIIGSLG	SEQ ID NO:110

O91262	MGSMCIAMAPRTLLLLIGCQLALG	SEQ ID NO:111
P0C0V9	MLSLILFFPSFAFA	SEQ ID NO:112
P30215	MFLLPRFILVSCIIGSLG	SEQ ID NO:113
Q14EB1	MLIIFLFFNFCYG	SEQ ID NO:114
Q9IKD2	MGRMCIAMAPRTLLLLIGCQLVFG	SEQ ID NO:115
Q70KP1	MLRMRVRPPSAIPVFLIFVLLPFVLTS	SEQ ID NO:116
Q5MQD1	MLIIFLFFYFCYG	SEQ ID NO:117
P31614	MCIAMAPRTLLLLIGCQLV	SEQ ID NO:118
O92367	MARTDAMAPRTLLLVLSLGYAFG	SEQ ID NO:119
Q8JSP9	MFLLPRFCLVCSIISTFG	SEQ ID NO:120
Q83356	MGSTCIAMAPRTLLLLIGCQLV	SEQ ID NO:121
Q8BB26	MFLLPRFCLVCSIIGTFG	SEQ ID NO:122
P68762	MFFSLLLMLGLTEA	SEQ ID NO:123
P07975	MFFSLLLVLGLTEA	SEQ ID NO:124
P87691	MLGLTEA	SEQ ID NO:125

Therefore, in some embodiments, the disclosed fusion protein has the amino acid sequence MEAKLFVLFC AFTALKAMGT NLSVPNPLGF FPDHQLDPAF GANSNNPDWD FNPIKDHWPA ANQVGVGAFG PGLTPPHGGI LGWSPQAQGI LTTVSTIPPP ASTNRQSGRQ PTPISPPLRD SHPQAMQWNS TAFHQALQDP RVRGLYLPAG GSSSGTVNPA PNIASHISSI SARTGDPVTN KLESVGVHQI LAIYSTVASS LVLLVSLGAI SFWMCSNGSL QCRICI (SEQ ID NO:2), or a conservative variant thereof having at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98%, 99%, or 100% sequence identity to SEQ ID NO:2 (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitutions).

Also disclosed are polynucleotides comprising nucleic acid sequences encoding the disclosed fusion proteins. For example, the nucleic acid sequences can be operably linked to expression control sequences. Thus, also disclosed are expression vectors for producing the disclosed fusion proteins as well as cells containing these polynucleotides and vectors for replicating the polynucleotides and vectors or to produce the disclose fusion proteins and/or VLPs. Therefore, the disclosed cell can also contain nucleic acid sequences encoding the disclosed fusion protein, including a vector comprising the nucleic acid sequences encoding the disclosed fusion protein.

Also disclosed are polynucleotides comprising nucleic acid sequences encoding the influenza virus M1 protein. For example, the nucleic acid sequences can be operably linked to expression control sequences. Thus, also disclosed are

expression vectors for producing the disclosed fusion proteins as well as cells containing these polynucleotides and vectors for replicating the polynucleotides and vectors or to produce the disclose fusion proteins and/or VLPs. Therefore, the disclosed cell can also contain nucleic acid sequences encoding the M1 protein, including a vector comprising the nucleic acid sequences encoding the M1 protein.

Also disclosed is a dual vector comprising a first nucleic acid sequence encoding a disclosed fusion protein and a second nucleic acid sequence encoding an M1 protein. The cell can be a prokaryotic or eukaryotic cell. For example, the cell can be a bacterium, an insect cell, a yeast cell, or a mammalian cell. The cell can be a human cell. Suitable vectors can be routinely selected based on the choice of cell used to produce the VLP. For example, where insect cells are used, suitable vectors include baculoviruses. In case of mammalian cells, plasmids for protein expression may be used.

Fusion proteins, also known as chimeric proteins, are proteins created through the joining of two or more genes which originally coded for separate proteins. Translation of this fusion gene results in a single polypeptide with function properties derived from each of the original proteins. Recombinant fusion proteins can be created artificially by recombinant DNA technology for use in biological research or therapeutics.

The functionality of fusion proteins is made possible by the fact that many protein functional domains are modular. In other words, the linear portion of a polypeptide which corresponds to a given domain, such as a tyrosine kinase domain, may be removed from the rest of the protein without destroying its intrinsic enzymatic capability. Thus, any of the herein disclosed functional domains can be used to design a fusion protein.

A recombinant fusion protein is a protein created through genetic engineering of a fusion gene. This typically involves removing the stop codon from a cDNA sequence coding for the first protein, then appending the cDNA sequence of the second protein in frame through ligation or overlap extension PCR. That DNA sequence will then be expressed by a cell as a single protein. The protein can be engineered to include the full sequence of both original proteins, or only a portion of either.

If the two entities are proteins, often linker (or "spacer") peptides are also added which make it more likely that the proteins fold independently and behave as expected. Especially in the case where the linkers enable protein purification, linkers in protein or peptide fusions are sometimes engineered with cleavage sites for

proteases or chemical agents which enable the liberation of the two separate proteins.

Virus Like Particles (VLPs)

The disclosed constructs may be expressed on the surface of a particle to mimic the natural conformation of preS on hepatitis B virions. For example, the disclosed fusion proteins may be incorporated into virus-like particles (VLPs) by including within the fusion protein a membrane anchor domain, such as a transmembrane domain and optional cytoplasmic domain of a viral envelope protein.

Non-replicating VLPs resemble infectious virus particles in structure and morphology, and contain immunologically relevant viral structural proteins. VLPs have been produced from both non-enveloped and enveloped viruses. Envelopes of VLPs are derived from the host cells similar to the way as enveloped viruses such as influenza A virus obtain their lipid envelopes from their host cells. Therefore, membrane-anchored proteins on the surfaces of enveloped viruses will be expressed in a native-like conformation if they are expressed in a membrane-anchored form.

Influenza VLPs involve lipid bilayers and host cell membrane proteins (Song, J. M., et al. J Proteome Res 2011 10:3450-3459). For example, Influenza VLPs containing the wild type M2 protein have been described (Song, J. M., et al. Proc Natl Acad Sci U S A 2011 108:757-761; Song, J. M., et al. PLoS One 2011 6:e14538). Enveloped VLPs may be composed of influenza matrix 1 (M1) protein as a particle forming core. These VLPs are produced, for example, by coinfecting insect cells with one or more recombinant baculoviruses co-expressing M1 proteins and the disclosed fusion proteins, culturing the insect cells under physiological conditions, and purifying the VLPs from insect cell culture supernatants.

Vaccine Compositions

Disclosed are vaccine compositions that comprise one or more of the fusion proteins described above. Although not required, the vaccine compositions optionally contain one or more immunostimulants. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody or cell-mediated) to an exogenous antigen. One preferred type of immunostimulant is an adjuvant.

Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, Bortadella pertussis or Mycobacterium tuberculosis derived proteins. The adjuvant may be a submicron oil-in-water emulsion of a metabolizable oil and an emulsifying agent. For example, the adjuvant

may comprise MF59™, which is a sub-micron oil-in-water emulsion of a squalene, polyoxyethylene sorbitan monooleate (Tween™ 80) and sorbitan trioleate. The adjuvant may also be a combination of the TLR4 agonist MPL (3-O-desacyl-4′-monophosphoryl lipid A) and aluminum salt, e.g., AS04 (GlaxoSmithKline, Philadelphia, Pa.).

Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Mich.); Merck Adjuvant 65 (Merck and Company, Rahway, N.J.); AS01, AS02, AS03, and AS04 (GlaxoSmithKline, Philadelphia, Pa.); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

The adjuvant composition can be a composition that induces an anti-inflammatory immune response (antibody or cell-mediated). Accordingly, high levels of anti-inflammatory cytokines (anti-inflammatory cytokines may include, but are not limited to, interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), and transforming growth factor beta (TGFβ). Optionally, an anti-inflammatory response would be mediated by CD4+ T helper cells. Bacterial flagellin has been shown to have adjuvant activity (McSorley *et al.*, J. Immunol. 169:3914-19, 2002). Also disclosed are polypeptide sequences that encode flagellin proteins that can be used in adjuvant compositions.

Optionally, the adjuvants increase lipopolysaccharide (LPS) responsiveness. Illustrative adjuvants include but are not limited to, monophosphoryl lipid A (MPL), aminoalkyl glucosaminide 4-phosphates (AGPs), including, but not limited to RC-512, RC-522, RC-527, RC-529, RC-544, and RC-560 (Corixa, Hamilton, Mont.).

In addition, the adjuvant composition can be one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (e.g., IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (e.g., IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a subject will support an immune response that includes Th1- and Th2-type responses. Optionally, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using

standard assays. Certain adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt adjuvants are available from Corixa Corporation (Seattle, Wash.). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Another adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, Mass.); Escin; Digitonin; or Gypsophila or Chenopodium quinoa saponins.

Additional illustrative adjuvants for use in the disclosed vaccine compositions include Montamide ISA 720 (Seppic, France), SAF (Chiron, Calif., United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (e.g., SBAS-2 or SBAS-4, available from GlaxoSmithKline, Philadelphia, Pa.), Detox (Enhanzyn™) (Corixa, Hamilton, Mont.), RC-529 (Corixa, Hamilton, Mont.) and other aminoalkyl glucosaminide 4-phosphates (AGPs).

In some embodiments, the adjuvant is incorporated into the VLP in a membrane-anchored form. For example, GM-CSF or a bacterial flagellin protein containing a membrane anchor can be incorporated into the disclosed VLPs.

Pharmaceutical Compositions

The disclosed vaccines can be used therapeutically in combination with a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). Suitable carriers and their formulations are described in Remington: The Science and Practice of Pharmacy (22nd ed.) eds. Loyd V. Allen, Jr., et al., Pharmaceutical Press, 2012. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody,

which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of vaccines to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the vaccine. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

The disclosed vaccines are preferably formulated for delivery via intranasal, intramuscular, subcutaneous, transdermal or sublingual administration.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium

hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

The disclosed vaccine can be used to supplement existing human vaccines to improve cross protection. Therefore, the disclosed vaccine can further include (or be administered in combination with) a whole inactivated virus, split viral vaccine, live attenuated hepatitis B vaccine, or another hepatitis B virus-like particle (VLP) vaccine, as well as DNA vaccines. For example, the disclosed vaccine can be combined with a hepatitis S vaccine.

The disclosed vaccine can further include (or be administered in combination with) one or more of classes of antibiotics, steroids, analgesics, anti-inflammatory agents, anti-histaminic agents, or any combination thereof. Antibiotics include Aminoglycosides, Cephalosporins, Chloramphenicol, Clindamycin, Erythromycins, Fluoroquinolones, Macrolides, Azolides, Metronidazole, Penicillins, Tetracyclines, Trimethoprim-sulfamethoxazole, and Vancomycin. Suitable steroids include andranes, such as testosterone. Narcotic and non-narcotic analgesics include morphine, codeine, heroin, hydromorphone, levorphanol, meperidine, methadone, oxydone, propoxyphene, fentanyl, methadone, naloxone, buprenorphine, butorphanol, nalbuphine, and pentazocine. Anti-inflammatory agents include alclofenac, alclometasone dipropionate, algestone acetonide, alpha amylase, amcinafal, amcinafide, amfenac sodium, amiprilose hydrochloride, anakinra, anirolac, anitrazafen, apazone, balsalazide disodium, bendazac, benoxaprofen, benzydamine hydrochloride, bromelains, broperamole, budesonide, carprofen, ciclopropfen, cintazone, cliprofen, clobetasol propionate, clobetasone butyrate, clopirac, cloticasone propionate, cormethasone acetate, cortodoxone, decanoate, deflazacort, delatestryl, depo-testosterone, desonide, desoximetasone, dexamethasone dipropionate, diclofenac potassium, diclofenac sodium, diflorasone diacetate, diflumidone sodium, diflunisal, difluprednate, diftalone, dimethyl sulfoxide, drocinonide, endrysone, enlimomab, enolicam sodium, epirizole, etodolac, etofenamate, felbinac, fenamole, fenbufen, fenclofenac, fenclorac, fendosal, fenpipalone, fentiazac, flazalone, fluazacort, flufenamic acid, flumizole, flunisolide acetate, flunixin, flunixin meglumine, fluocortin butyl, fluorometholone acetate, fluquazone, flurbiprofen, fluretofen, fluticasone propionate, furaprofen, furobufen, halcinonide, halobetasol propionate, halopredone acetate, ibufenac, ibuprofen, ibuprofen aluminum, ibuprofen piconol, ilonidap, indomethacin, indomethacin sodium, indoprofen, indoxole, intrazole, isoflupredone acetate, isoxepac, isoxicam, ketoprofen, lofemizole hydrochloride, lomoxicam, loteprednol etabonate,

meclofenamate sodium, meclofenamic acid, meclorisone dibutyrate, mefenamic acid, mesalamine, meseclazone, mesterolone, methandrostenolone, methenolone, methenolone acetate, methylprednisolone suleptanate, momiflumate, nabumetone, nandrolone, naproxen, naproxen sodium, naproxol, nimazone, olsalazine sodium, orgotein, orpanoxin, oxandrolane, oxaprozin, oxyphenbutazone, oxymetholone, paranyline hydrochloride, pentosan polysulfate sodium, phenbutazone sodium glycerate, pirfenidone, piroxicam, piroxicam cinnamate, piroxicam olamine, pirprofen, prednazate, prifelone, prodolic acid, proquazone, proxazole, proxazole citrate, rimexolone, romazarit, salcolex, salnacedin, salsalate, sanguinarium chloride, seclazone, sermetacin, stanozolol, sudoxicam, sulindac, suprofen, talmetacin, talniflumate, talosalate, tebufelone, tenidap, tenidap sodium, tenoxicam, tesicam, tesimide, testosterone, testosterone blends, tetrydamine, tiopinac, tixocortol pivalate, tolmetin, tolmetin sodium, triclonide, triflumidate, zidometacin, and zomepirac sodium. Anti-histaminic agents include ethanolamines (e.g., diphenhydrmine carbinoxamine), Ethylenediamine (e.g., tripelennamine pyrilamine), Alkylamine (e.g., chlorpheniramine, dexchlorpheniramine, brompheniramine, triprolidine), other anti-histamines like astemizole, loratadine, fexofenadine, bropheniramine, clemastine, acetaminophen, pseudoephedrine, triprolidine).

Methods of Vaccinating a Subject

A method of vaccinating a subject for hepatitis B is disclosed that involves administering the disclosed vaccine to a subject in need thereof. The disclosed vaccine may be administered in a number of ways. For example, the disclosed vaccine can be administered intramuscularly, intranasally, or by microneedle in the skin. The compositions may be administered orally, intravenously, subcutaneously, transdermally (e.g., by microneedle), intraperitoneally, ophthalmically, vaginally, rectally, sublingually, or by inhalation.

Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained.

The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an

exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. For example, effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are affected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. A typical dosage of the disclosed vaccine used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per vaccination, such as 10 µg/kg to 50 mg/kg, or 50 µg/kg to 10 mg/kg, depending on the factors mentioned above.

T Cell Expansion for Adoptive Cell Transfer

Also disclosed is a method for activating and expanding CD8+ T cells for adoptive cell transfer (ACT). The method generally involves co-culturing CD8+ T cells and dendritic cells with the VLPs disclosed herein.

ACT may be performed by (i) obtaining autologous lymphocytes from a mammal, (ii) culturing the autologous lymphocytes to produce expanded lymphocytes, and (ii) administering the expanded lymphocytes to the mammal. Preferably, the lymphocytes are isolated from the mammal to be treated, i.e. autologous transfer.

Expanded lymphocytes produced by the disclosed methods can be administered as an intra-arterial or intravenous infusion, which preferably lasts about 30 to about 60 minutes. Other examples of routes of administration include intraperitoneal, intrathecal and intralymphatic. Likewise, any suitable dose of lymphocytes can be administered. In one embodiment, about 1×10^{10} lymphocytes to about 15×10^{10} lymphocytes are administered.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without

departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

EXAMPLES

Example 1: A virus-like particle vaccine of the hepatitis B virus preS antigen protects mice against challenge.

There are three envelope proteins in the HBV virion, S, M and L. The preS protein is part of the L protein, a 163 amino acid extension at the N-terminus of the S protein (genotype A). preS may be further divided as preS1 (a.a.1-108) and preS2 (a.a.109-163). In the M protein, only preS2 is present at the N-terminus in addition to the amino acids that are common among the S, M and L proteins (Churin Y, et al. Hepatobiliary Surg Nutr 2015 4:1-10). Two well-known functions are related to preS. First of all, preS includes the region that interacts with the specific host receptor (Yan H, et al. Elife 2012 1:e00049). Further studies define the interaction motif to be within the first 48 amino acids of preS (Barrera A, et al. J Virol 2005 79:9786-9798; Glebe D, et al. Gastroenterology 2005 129:234-245; Gripon P, et al. J Virol 2005 79:1613-1622). The other important function of preS is that in the preS region, there are highly immunogenic sites as B and T cell epitopes (Vento S, et al. Immunology 1987 62:593-598). It has been reported that preS could induce humoral responses in mice which were nonresponsive to the S antigen, indicating that preS represents a potential antigen for novel HBV vaccine candidates (Milich DR. Immunol Today 1988 9:380-386). Notably, humoral response may play a major role in preventing HBV spread to uninfected cells. Besides, it is generally believed that a proper $CD4^+$ helper T cell response is a prerequisite for an adequate humoral response (Celis E, et al. J Immunol 1984 132:1511-1516). Furthermore, T cell responses may help extend the longevity of humoral immunity (Bauer T, et al. Vaccine 2006 24:572-577; Wiegand J, et al. J Viral Hepat 2010 17:631-639). However, how these preS epitopes are related to virus clearance is not very clear. It is widely accepted that the $CD8^+$ T cell response is primarily responsible for HBV clearance in both cytopathic and noncytopathic manner (Chisari FV, et al. Pathol Biol 2010 58:258-266). More recent studies demonstrate that HBV-specific $CD8^+$ T cells are able to clear HBV-infected hepatocytes by secretion of antiviral cytokines, such as IFN-γ, and TNF-α (Kosinska AD, et al. Hepat Res Treat 2010 2010:817580).

Virus-like particles (VLPs) resemble authentic native viruses in structure and morphology, but are non-infectious, because they assemble without containing genetic material. Compared to individual proteins or peptides, VLPs significantly

improve humoral responses by presenting conformational epitopes more similar to the native virus. Owing to their highly repetitive surface, VLPs are capable of eliciting robust B cell responses in the absence of adjuvants by efficiently cross-linking specific receptors on B cells (Roldao A, et al. Expert Rev Vaccines 2010 9:1149-1176). Besides, VLPs could also induce potent cytotoxic T lymphocyte (CTL) responses in immunized animals (Liu XS, et al. Virology 1998 252:39-45).

A virus-like particle (preS VLP) that contains the matrix protein M1 from influenza virus and the transmembrane domain and cytoplasmic tail of influenza virus hemagglutinin (HA) to form the scaffold was produced. The HBV preS antigen was presented on the surface of VLP by fusing it with the HA fragment. The immunogenicity of preS VLP was assessed as a potential vaccine candidate. Immunization with preS VLP induced both potent humoral and cellular immune responses, and protected mice from HBV challenge.

Materials and methods

Plasmids and cells

The vector for expressing HBV preS (adw subtype, Accession Number AGW20902) in *E. coli* (pET28b-preS) was constructed previously (Lian M, et al. Virol J 2007 4:93). The His-tagged preS protein was expressed and purified as described previously (Lian M, et al. Virol J 2007 4:93). The plasmids for expressing M1 protein (the matrix protein) of influenza virus A/sw/Spain/53207/04 and a preS-HA (HA=hemagglutinin) chimeric protein were constructed by inserting the coding sequence in pCAGGS. The amino acid 41 of M1 was mutated to Ala (pCAGGS-M1). The preS-HA has the sequence of HBV preS followed by aa521-566 of HA (pCAGGS-preS-HA). 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS).

Indirect immunofluorescence

293T cells were grown on glass coverslips and transfected with pCAGGS-M1 and pCAGGS-preS-HA. 48 hr posttransfection, cells were fixed with 4% paraformaldehyde. Cells were classified into two groups. One was permeabilized with 0.2% Triton X-100 for 5 min, the other without permeabilization. After blocking for 1h in PBS containing 5% goat serum, all cells were incubated with polyclonal rabbit anti-preS sera at 4°C overnight. Cells were washed with PBS following incubation with Alexa Fluor® 488-Conjugated goat anti-rabbit secondary antibody for 1 h at 37°C. After washing, cells were stained with DAPI for 10 min, and then mounted onto microscope slides. Confocal slices were acquired with a 100× objective, using a Zeiss 510 confocal microscope with random sampling.

Preparation and characterization of the virus-like particles

The pCAGGS-M1 and pCAGGS-preS-HA plasmids were transfected into 293T cells with polyethylenimine. 72 hr after transfection, the culture medium was centrifuged at 6000 rpm for 15 min at 4°C to remove cellular debris, followed by centrifugation at 22,000 rpm for 3 hr at 4°C. The pellet was resuspended in PBS at 4°C overnight, and further purified through a 20%-60% sucrose gradient in a Beckman SW41Ti rotor at 30,000 rpm for 3 hr at 4°C. The 40% sucrose fraction was harvested and diluted with PBS by about 5 fold. After centrifugation at 22,000 rpm for 3 hr at 4°C to remove the sucrose, the virus-like particles were resuspended in PBS at 4°C overnight. A sample was applied to a 400 mesh carbon-coated copper grid, and stained with 1% phosphotungstic acid (J&K Scientific). preS VLP was visualized on a Tecnai G^2 Spirit transmission election microscope operating at 120 kV.

LC-MS/MS analysis

The expression of M1 and preS-HA was analysed by LC-MS/MS. Briefly, 40% sucrose fraction were subjected to electrophoresis on a 12%-SDS-PAGE gel, which was stained by coomassie R250. The coomassie R250 stained gel bands were cut, followed by in-gel digestion with trypsin [Promega, enzyme: protein= 1:50 (wt/wt)] at 37 °C for 12 h in 25 mM ammonium bicarbonate buffer. The lyophilized tryptic digested samples were re-dissolved in 2% acetonitrile, 0.1% formic acid, and loaded on ChromXP C18 (3 µm, 120 Å) nanoLC trap column. The online trapping, desalting procedure was carried out at a flow rate of 2 µL/min for 10 min with 100% solvent A (Solvent A: water/acetonitrile/formic acid = 98/2/0.1% solvent B: 2/98/0.1%). Then, an 60-min gradient elution ranging from 5-35% acetonitrile (0.1% formic acid) was used on an analytical column (75 µm x 15 cm C18- 3µm 120 Å, ChromXP Eksigent). LC-MS/MS analysis was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON). Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 5 PSI, and an interface heater temperature of 150 °C. The MS was operated with TOF-MS scans. For IDA, survey scans were acquired in 250 ms and as many as 25 product ion scans (90 ms) were collected if exceeding a threshold of 150 counts per second (counts/s) and with a +2 to +4 charge-state. A Rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for ½ of peak width (~12 s). For data analysis, the .wiff files were processed by ProteinPilot 5.0. Searches were performed against the local database including the protein sequences for M1 and preS-HA, using the default settings.

Immunization and challenge

Female Balb/c mice of 6-8 weeks old were immunized by injecting the antigen preparation in the hindlimb. A booster was given on day 22. Blood was collected on day 52, and 112, and neutralizing antibody titers were determined by ELISA. On day 52, activated T cells in splenocytes or intrahepatic leukocytes were analysed by ELISPOT and FACS. The immunized mice were challenged on day 70. 10 μg of pT-HBV1.3 (a plasmid containing 1.3 genome length of HBV) was injected under hydrodynamic conditions to establish HBV infection as previously described (Yang PL, et al. Proc Natl Acad Sci U S A 2002 99:13825-13830). Hydrodynamic injection of viral DNA is an accepted mouse model of acute hepatitis B virus infection. Blood samples were collected at different time points to measure HBV antigens. On day 67, mice were sacrificed and liver tissues were used for measuring antigens and RNA of HBV. Activated T cells were also analysed by FACS and ELISPOT assay. All mouse experiments were conducted in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Committee for Animal Experiments at Peking University.

Immunization in HBV transgenic mice

All mouse experiments were performed in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Institutional Animal Care and Use Committee of Peking University. Six- to 8-week-old female HBV transgenic mice (ayw subtype) were obtained from Infectious Disease Center of No. 458 Hospital (Guangzhou, China). HBV transgenic mice were immunized intramuscularly with 20 μg of preS VLP in the hindlimb, and were boosted on days 22 and 43, respectively. Blood was collected on days 0, 30 and 70, and anti-preS antibody titers were determined by ELISA. On day 70, activated T cells in splenocytes were analyzed by ELISPOT and flow cytometry. All mouse experiments were conducted in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Committee for Animal Experiments at Peking University.

Isolation of splenocytes and intrahepatic leukocytes

For the isolation of splenocytes, splenocytes were gently grinded followed by passaging through 40 μm strainers and treating with ACK lysing buffer. After washing with PBS, cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin–L-Glutamine. For the isolation of intrahepatic leukocytes, mice livers were perfused with pre-warmed Hanks' balanced solution without Ca^{2+}, Mg^{2+}, followed by perfusing with 20 mL 0.025% collagenase D in Hanks' balanced salt solution, and let sit for 10 min at 37°C. Livers were then

gently grinded followed by passaging through 40 μm strainers. After centrifugation, cells were resuspended in 40% (vol/vol) Percoll in DMEM, and layered over 70% Percoll in PBS (vol/vol). After centrifugation of the gradient for 20 min at 2000 rpm, the cells at the interphase were collected. The cells were then treated with ACK lysing buffer, washed with PBS, and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin–L-Glutamine for further analysis.

Enzyme-linked immunospot assay

T cell responses were determined using an IFN-γ ELISPOT set (BD Biosciences) following the manufacturer's protocol. Briefly, 96-well plates were coated with purified anti-mouse IFN-γ antibody (1:200) at 4°C overnight, and then were blocked for 2 h using DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin– L-Glutamine. Splenocytes or intrahepatic leukocytes were seeded at 2×10^5/well. Peptides representing previously described epitopes present in preS (Table 1) or purified preS protein were used to stimulate cells for 36 h at 37°C in a 5% CO_2 and humidified incubator, with media and phorbol myristate acetate (PMA)/ionomycin-treated cells used as negative and positive controls, respectively. After being washed, cells were incubated with biotinylated anti-mouse IFN-γ antibody (1:250) for 2 h at room temperature, and then incubated with streptavidin-horseradish peroxidase (HRP) (1:1,000) for 1 h. Following the final washes, 3-amino-9-ethylcarbazole (AEC) substrate (Alfa Aesar) was added to the wells and allowed to develop at room temperature for 40 min. The reaction was stopped with distilled water, and the plates were allowed to air dry before spot-forming cells were enumerated by using an ELISPOT plate reader.

Table 2. preS-specific T cell epitopes.

Epitope	Residues	Amino acid sequence	Reference
1	preS1 10-19	PLGFFPDHQL (SEQ ID NO:12)	*
2	preS1 41-56	WPAANQVGVGAFGPGL (SEQ ID NO:13)	**
3	preS2 109-134	MQWNSTAFHQALQDPRVRGLYLPAGG (SEQ ID NO:14)	***

* Ferrari C, et al. Gastroenterology 1992 103:255-263.
** Doh H, et al. FEMS Immunol Med Microbiol 2003 35:77-85.
*** Pajot A, et al. Microbes Infect 2006 8:2783-2790

Flow cytometry

Splenocytes or intrahepatic leukocytes were resuspended in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin– L-Glutamine, and then were seeded at 2×10^6 /well. The cells were then stimulated for 6

h with preS-specific peptides or purified recombinant preS diluted to a final concentration of 10 μg/ml in DMEM supplemented with 2 μg/ml brefeldin A (BD Biosciences). The cells were then washed in staining buffer (PBS containing 2% fetal bovine serum) and stained for CD8 and CD4 surface expression for 30 min at 4°C using fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 antibody (BD Biosciences) and peridinin chlorophyll protein(PerCP)-conjugated anti-mouse CD4 antibody(BD Biosciences). Then the cells were washed, fixed, and permeabilized using a commercially available Cytofix/Cytoperm kit (BD Biosciences). The cells were then stained for 40 min at 4°C for intracellular cytokine expression using phycoerythrin (PE)-conjugated anti-mouse IFN-γ antibody (BD Biosciences). After washing, cells were resuspended in staining buffer and analysed using a BD FACS CantoTM II flow cytometer (BD Biosciences) and FACSDiva Version 6.1.3. Results were generated from data gathered from 200,000 cells.

ELISA

Purified preS antigen (5μg/ml) or preS VLP (1μg/ml) was absorbed to 96 well plates, blocked with 10% BSA, and then 50 μl of 1:100 dilution of sera was incubated for 30 min at 37°C followed by incubation with added HRP-conjugated anti-mouse IgG, IgG1 or IgG2a (Santa Cruz Biotechnology) for 30 min at 37°C, and then with TMB substrate for 10 minutes before stopping with 2 M H_2SO_4 for measurement of optical density at 450 nm. In addition, serum samples were diluted 1:5 for HBsAg ang HBeAg detection.

Neutralization

For infection experiments, 5×10^5 HepG2/hNTCP cells per well were cultured in 24-well plate and incubated overnight with the viral inoculum (M.O.I. of 500) alone or together with various dilutions of mouse anti-preS VLP sera or 1,000 fold dilution of hepatitis B immunoglobulin (HBIG) (from Chengdu Rongsheng Bioproduct Company, with a protein concentration of 144 mg/ml), with 4% PEG present during virus infection. Medium was changed every 2 or 3 days, and HBeAg was measured at 1 week post infection using Diagnostic ELISA Kit for Hepatitis B e antigen (Kehua Bio-engineering).

qPCR analysis

For detecting mRNA in 293T cells transfected with expression vectors, 48 hr posttransfection, total RNAs from 293T cells were extracted using the QIAGEN RNeasy Mini Kit following the manufacturer's instructions. Total RNAs were stored at -20°C until being used. Any possible contaminating DNA was erased by DNase I (Takara). Total RNAs were quantified using the NanoDrop 2000 spectrophotometer

(Thermo Scientific). 1 μg total RNAs were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 μL reaction as previously described (Zheng W, et al. Cell Chem Biol 2016 23:1002-1013). cDNA derived from 5 ng total RNAs was used as template for real-time PCR amplification. Primers (preS-HA-FW: 5'-CCACCAATCGGCAGTC-3' (SEQ ID NO:4)) and (preS-HA-RV: 5'-GCCACCAGCAGGAAGAT-3' (SEQ ID NO:5)) were used for preS-HA transcripts; (M1-FW: 5'-TGACAACAACCAACCCACT-3' (SEQ ID NO:6)) and (M1-RV: 5'-CTGCTGCTTGCTCACTCG-3' (SEQ ID NO:7)) were for M1 transcripts; (β-actin-FW: 5'-TCATGAAGTGTGACGTGGACATC-3' (SEQ ID NO:8)) and (β-actin-RV: 5'-CAGGAGGAGCAATGATCTTGATCT-3' (SEQ ID NO:9)) were used for β-actin transcripts. qRT-PCR was performed with GoTaq qPCR Master Mix (Promega) following the manufacturer's protocol for a total reaction volume of 20 μL in the CFX96 Real-Time PCR Detection System (Bio-Rad). The reaction product was subjected to agarose gel electrophoresis.

For challenge studies, total RNAs from the liver of HBV-challenged mice were isolated with Trizol reagent (Invitrogen). Then chloroform was added and mixed. After centrifugation, the aqueous layer was carefully collected and mixed with isopropanol and let sit for 10 min. After centrifugation, the supernatant was aspirated, and the precipitation was washed with 70% ethanol, followed by dissolved with nuclease-free water. Any possible contaminating DNA was erased by DNase I (Takara). Total RNAs were quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific). 1 μg total RNAs were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 μL reaction as previously described (Zheng W, et al. Cell Chem Biol 2016 23:1002-1013). cDNA derived from 2 ng total RNAs was used as template for real-time PCR amplification. Primers (HBV2270FW: 5'-GAGTGTGGATTCGCACTCC-3' (SEQ ID NO:10)) and (HBV2392RV: 5'-GAGGCGAGGGAGTTCTTCT-3' (SEQ ID NO:11)) were used for HBV RNA transcripts (Yan H, et al. Elife 2012 1:e00049). qRT-PCR was conducted with GoTaq qPCR Master Mix (Promega) following the manufacturer's instructions for a total reaction volume of 20 μL in the CFX96 Real-Time PCR Detection System (Bio-Rad).

Immunohistochemistry

Liver tissue was collected and fixed in 10% neutral formalin. After paraffin embedding, liver sections were used to detect HBV core antigen by immunohistochemical staining using polyclonal rabbit anti-HBcAg antibody (Dako).

Results

Construction and preparation of preS VLP

Co-expression of influenza virus M1 and HA releases a virus-like particle decorated with HA antigen (Galarza JM, et al. Viral Immunol 2005 18:244-251). In order to generate a virus-like particle that is decorated with HBV preS antigen, a chimeric protein was constructed that has the preS sequence fused at the N-terminus of a HA fragment that includes its transmembrane domain and the cytoplasmic tail (Fig. 1A). The signal peptide from HA was also added in front of the preS sequence which may be removed after the chimeric protein is expressed. The amino acid 41 of M1 was mutated to Ala to enhance the release of the virus-like particle (Campbell PJ, et al. J Virol 2014 88:7569-7577). After transfection of 293T cells with pCAGGS-M1, pCAGGS-preS-HA, or both plasmids, respectively, total RNAs were extracted by QIAGEN RNeasy Mini Kit and analysed for the transcription levels of M1 and preS-HA. The qPCR results suggested that both M1 and preS-HA genes had been transcribed adequately in 293T cells 48 hr after transfection (Fig. 1B). After co-transfection of pCAGGS vectors expressing M1 and preS-HA, respectively, into 293T cells, expression of the preS antigen was readily detected on the cellular membrane by immunofluorescent microscopy (Fig. 1C and 1D). This is consistent with the construction of the preS-HA chimeric protein because the transmembrane domain of HA retains the chimeric protein in the cellular membrane. If the M1 protein was not co-expressed, the preS-HA chimeric protein appeared to be unable to expose the preS antigen on the exterior of the cellular membrane because the preS antigen was only detectable after the cellular membrane was permeabilized with Triton X-100. Western blot analysis also confirmed the expression of preS-HA in 293T cells (Fig. 2). Interestingly, the presence of preS-HA was detected in the supernatant only when the M1 protein was co-expressed, suggesting that M1 is required for the secretion of preS-HA from cells (Fig. 2A).

Purification and characterization of preS VLP

The culture media were collected from the cells that were cotransfected with pCAGGS-M1 and pCAGGS-preS-HA at 72 hr after transfection. The culture media were laid on a sucrose gradient and subjected to ultracentrifugation. A sample was collected from the 40% sucrose fraction. By SDS-PAGE and western blot analysis, the protein corresponding to the preS-HA antigen was identified (Fig. 2B and 2C). A negative stain electron micrograph showed that this sample contains virus-like particles (Fig. 2D). The sample was further characterized by LC-MS/MS (Fig. 2E). The M1 sequence identified by MS analysis covered above 90% of the full length M1

sequence. However, only two peptides with high confidence (≥95%) in preS-HA were identified, which may be resulted from the glycosylation of preS (Lambert C, et al. Virol J 2007 4:45). These data showed that VLPs composed of M1 and preS-HA proteins were successfully purified.

Immunization with preS VLP

Balb/c mice were immunized each with 10 μg of the preS antigen as VLP, or recombinant preS purified from *E. coli* expression (Lian M, et al. Virol J 2007 4:93) with alum adjuvant, respectively. PBS was also used as a blank control. A booster was given on day 22. A blood sample was collected from each mouse on day 52 and 112, and sera were prepared from these samples. All sera samples were diluted by 100 fold and the serum antibody titers were determined by ELISA as shown in Fig. 3. The data revealed that VLP is a more potent preS antigen than recombinant preS protein in generating anti-preS neutralizing antibodies, even without the use of any adjuvant. In particular, preS VLP elicited high level total IgG including both anti-preS IgG1 (Th2 isotype) and IgG2a (Th1 isotype).

To further test whether anti-preS VLP sera could block HBV infection of human hepatocytes, in vitro infection experiments were conducted. Using hepatitis B immunoglobulin (HBIG) as a positive control, the anti-preS VLP sera clearly prevented HBV from infecting HepG2/hNTCP cells, as demonstrated by a decreased level of HBeAg in the supernatant of cell culture (Fig. 3E). Collectively, these results indicate that preS VLP can stimulate anti-preS neutralizing antibodies in mice.

T cell responses play a role in the induction of humoral immunity, and are crucial to effectiveness of a therapeutic HBV vaccine (Celis E, et al. J Immunol 1984 132:1511-1516; Chisari FV, et al. Pathol Biol 2010 58:258-266). To evaluate if preS-specific T cell responses were produced, T lymphocytes from spleen were isolated and cultured in culture plates. After stimulating with preS-specific T cell peptide epitopes (Table 2) for 6 hr, the T cells were analysed for CD4, CD8 and INF-γ by FACS. The results of activated T cells were shown in Fig. 4. In mice immunized with preS VLP, $CD4^+$ and $CD8^+$ T cells were much higher than either the controls or those immunized with recombinant preS. The number of $CD8^+$ T cells or $CD4^+$ T cells coincides with that of $CD8^+$ T cells or $CD4^+$ T cells producing intracellular INF-γ. The secretion of INF-γ, which is considered to be a key in controlling and clearing HBV replication, was analysed by ELISPOT. The results showed that preS-specific INF-γ production is more abundant when mice were immunized with preS VLP (Fig. 4E). This suggests that preS VLP could evoke potent preS-specific $CD8^+$ T cells that are capable of HBV clearance.

Vaccination with preS VLP offers protection against challenge

Immunized mice were challenged on day 70. HBV replication was induced by hydrodynamic injection of pT-HBV1.3 plasmid containing a 1.3-fold-overlength genome of HBV (10 μg). Liver tissues were collected on day 77 for HBV RNA detection, immunohistochemistry and T cell response analysis. HBV RNA copies were measured by qPCR (Fig. 5A). The levels of HBV RNA in preS VLP-immunized animals were significantly lower than that in animals immunized with recombinant preS. Liver sections stained for HBV core antigen indicated that HBcAg-positive hepatocytes in preS VLP-immunized animals eliminated almost entirely, while persisting at high levels in preS-immunized animals (Fig. 5B). A blood sample was collected from each mouse on day 70, 72, 74 and 77, and sera were prepared from these samples. HBsAg levels in preS VLP-immunized animals remained nearly undetectable through at least 7 days, while rising to high levels in animals immunized with recombinant preS on day 4 postchallenge (Fig. 5C). Levels of serum HBeAg also remained nearly undetectable in preS VLP-immunized animals over the course of 7 days. On the other hand, HBeAg levels in preS-immunized animals elevated on day 4 then dropped to nearly undetectable levels on day 7 postchallange (Fig. 5D). This phase of HBsAg and HBeAg clearance coincides with the development of anti-preS neutralizing antibodies (Fig. 5E). Thus, immunization with preS VLP controls and clears HBV replication within 7 days following HBV challenge by hydrodynamic injection of a 1.3-fold-overlength genome of HBV.

Protection effect correlates with memory T cell responses

Since CD8 T cell responses would likely play an important role in HBV clearance, T cell recall responses in the spleen were analysed by FACS (Fig. 6A-D). In mice immunized with preS VLP, the number of $CD8^+$ and $CD4^+$ T cells was obviously higher than the controls and those immunized with recombinant preS, and the number of $CD8^+$ T cells or $CD4^+$ T cells producing intracellular INF-γ was also higher, but not as much. Notably, the number of $CD8^+$ T cells was significantly higher when mice were immunized with preS VLP, indicating that preS VLP induced memory T cells that could be recalled upon HBV challenge, and that the T cells were responsible for clearance of HBV. Furthermore, the T cell recall response was also measured by IFN-γ ELISPOT assays 7 days postchallenge on both splenocytes (Fig. 6E) and intrahepatic leukocytes (Fig. 6F). Interestingly, preS-specific T cell responses present in the liver and spleen of preS VLP-immunized mice were more potent than those in recombinant preS-immunized mice, suggesting that preS-specific T cells mediate clearance of HBV from transfected hepatocytes. Taken

together, preS VLP elicits robust anti-preS neutralizing antibodies and preS-specific T cell responses, and could protect mice against HBV challenge, representing a novel prophylactic or potentially therapeutic vaccine candidate for HBV infection in humans.

Immune responses in HBV transgenic mice

The therapeutic potential of preS VLP immunization was investigated by employing HBV transgenic mice as a model of chronic infection. HBV transgenic mice were first primed with preS VLP or PBS, and then were boosted on days 22 and 43, respectively. Despite that HBV transgenic mice have become tolerance to HBV (Allweiss L, Dandri M. J Hepatol 2016;64:S17-31), preS VLP could still induce high levels of anti-preS total IgG including both anti-preS IgG1 (Th2 isotype) and IgG2a (Th1 isotype) (Fig. 7B-D), suggesting a balanced Th1/Th2 response against preS VLP. In addition, preS VLP immunization could also stimulate a higher percentage of preS-specific CD4+ and CD8+ T cells than either the control or recombinant preS immunization (Fig. 7E, and 7F). Furthermore, preS-specific IFN-γ-producing T cells are more plentiful in mice immunized with preS VLP than either the controls or those immunized with recombinant preS protein (Fig. 7G and 7H), implying the therapeutic potential of preS VLP. Taken together, preS VLP vaccine could induce preS-specific CD8+ and CD4+ T cell responses in HBV transgenic mice.

Discussion

Despite the success of currently available HBV S antigen-based vaccines, there are still 5-10% people do not respond adequately to provide protection against exposure to HBV (Kubba AK, et al. Commun Dis Public Health 2003 6:106-112). Due to the additional B and T cell epitopes in HBV preS region, preS represents an attractive antigen for HBV vaccine candidates that are able to overcome non-responsiveness to the S antigen-based vaccines (Grgacic EV, et al. Methods 2006 40:60-65). When combined with the S-antigen based vaccine, preS antigen may enhance the overall protection in a synergistic manner.

VLP-based vaccines are of high safety for lacking genetic material. Moreover, VLPs often display high immunogenicity because of presenting highly repetitive epitopes more similar to the native virus (Roldao A, et al. Expert Rev Vaccines 2010 9:1149-1176). Additionally, co-expression of influenza matrix protein M1 and hemagglutinin (HA) can self-assembly into virus-like particles (Galarza JM, et al. Viral Immunol 2005 18:244-251). This influenza VLP scaffold could be developed as a non-egg-based, cell culture-derived vaccine platform to present foreign antigens, such as HBV preS antigen (Lee DH, et al. Clin Exp Vaccine Res 2014 3:133-139).

A unique system has been successfully designed to produce a preS virus-like particle by co-expressing a M1 protein from influenza virus and a preS-HA chimeric protein. preS VLP in culture media may be purified by ultracentrifugation in a sucrose gradient. preS VLP is able to evoke much higher antibody titers than recombinant preS even without any adjuvants. This is likely due to the formation of HBV preS VLP, as this allows for endocytosis by antigen-presenting cells. Besides, the potential of preS VLP to elicit high antibody titers is most likely facilitated by highly repetitive preS antigen that is presented on the surface of the VLPs.

Anti-preS1 antibodies were previously found to be remarkably effective in neutralization of HBV (Neurath AR, et al. Vaccine 1989 7:234-236). The identification of protective epitopes within the preS1 region reveals that preS1 specific antibodies neutralize the virus by blocking the binding of host NTCP receptor (Yan H, et al. Elife 2012 1:e00049; Sankhyan A, et al. Sci Rep 2016 6:21240). These observations support the notion that an effective preS-based vaccine may expand the population of protected individuals. The disclosed results demonstrated that preS VLP can be an effective preS vaccine candidate. At the same time, as preS antigen is carried on the surface of VLP, class I antigen presentation pathway within the host cells can be exploited, resulting in potent T cell responses, which may also play a role in eliciting the high B cell responses. Furthermore, preS VLP are capable of protecting mice from HBV challenge, which may be probably due to the effective induction of B cell responses, and generation of memory $CD8^+$ T cells that are able to control infection.

The preS antigen has been exploited previously as candidates of prophylactic or therapeutic vaccines (Shouval D, et al. Med Microbiol Immunol 2015 204:57-68). When recombinant preS proteins were used in immunization, the antibody titers were not very high (Sylvan SP, et al. Vaccine 2009 28:446-451). The ability of neutralizing HBV by these antibodies is limited. More critically, no T cell responses against HBV were demonstrated (Raz R, et al. Vaccine 1996 14:207-211; Shapira MY, et al. J Hepatol 2001 34:123-127). This is consistent with that isolated proteins generally do not induce T cell responses because they usually are not internalized by antigen presenting cells (Pennock ND, et al. Trends in immunology 2016 37:170-180). In order to overcome the barrier, virus or yeast vectors were employed to express HBV antigens (Sallberg M, et al. Human gene therapy 1998 9:1719-1729; Martin P, et al. Gut 2015 64:1961-1971; Reynolds TD, et al. Journal of virology 2015 89:10407-10415; King TH, et al. PloS one 2014 9:e101904). However, there are safety concerns when a replicating vector is used despite their capability to induce strong T cell responses. In addition, these vectors may only be given once because the host

will establish immunity against the vector. The disclosed design of preS VLP has the advantages of mimicking the antigen surface of a virus particle and being able to enter the antigen presenting cells to elicit strong T cell responses, and at the same time, it permits multiple doses and does not replicate any foreign microorganism.

In conclusion, this preS VLP may be used as both prophylactic and therapeutic vaccines against hepatitis B virus infection in humans.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A fusion protein comprising a hepatitis B preS antigen fused at the N-terminus of a transmembrane domain and optional cytoplasmic tail of a viral envelope protein.

2. The fusion protein of claim 1, wherein the preS antigen comprises the amino acid sequence SEQ ID NO:1, or an antigenic variant thereof having at least 90% identity to SEQ ID NO:1.

3. The fusion protein of claim 1 or 2, wherein the viral envelope protein comprises a viral type I transmembrane glycoprotein.

4. The fusion protein of claim 3, wherein the viral envelope protein comprises an influenza virus hemagglutinin (HA) protein.

5. The fusion protein of claim 4, comprising the amino acid sequence SEQ ID NO:2.

6. The fusion protein of any one of claims 1 to 5, further comprising a signal peptide at the N-terminus.

7. The fusion protein of claim 6, wherein the signal peptide comprises the signal peptide from HA.

8. A virus like particle (VLP) comprising the fusion protein of any one of claims 1 to 5 and a viral matrix protein compatible with the type I transmembrane glycoprotein.

9. The VLP of claim 8, wherein the matrix protein comprises an influenza virus matrix protein 1 (M1).

10. The VLP of claim 8, produced by coinfecting insect cells with one or more recombinant baculoviruses expressing the matrix protein and the fusion protein of any one of claims 1 to 7, culturing the insect cells under physiological conditions, and purifying the VLPs from insect cell culture supernatants.

11. The VLP of claim 10, produced by introducing into mammalian cells one or more expression vectors expressing the matrix protein and the fusion protein of any one of claims 1 to 7, culturing the mammalian cells under appropriate conditions, and purifying the VLPs from cell culture supernatants.

12. A vaccine comprising an effective amount of the VLP of any one of claims 8 to 11 in a pharmaceutically acceptable excipient.

13. The vaccine of any one of claims 11 or 12, further comprising an adjuvant.

14. An isolated polynucleotide comprising a nucleic acid sequence encoding the fusion protein of any one of claims **Error! Reference source not found.** to 5.

15. The isolated polynucleotide of claim 14, wherein the nucleic acid sequence encoding the fusion protein is operably linked to an expression control sequence.

16. A cell comprising the isolated polynucleotide of claim 14 or 15.

17. The cell of claim 16 wherein the cell is a bacterium, insect cell, mammalian cell, or yeast cell.

18. A method of vaccinating a subject for hepatitis B comprising administering the vaccine of claim 12 or 13 to a subject in need thereof by intranasal, intramuscular, subcutaneous, transdermal, or sublingual administration.

19. The method of claim 18, further comprising administering to the subject a vaccine comprising the hepatitis B surface antigen (HBsAg).

20. A method for activating CD8+ T cells for adoptive cell therapy (ACT), comprising co-culturing CD8+ T cells and dendritic cells with the VLPs of any one of claims 8 to 11.

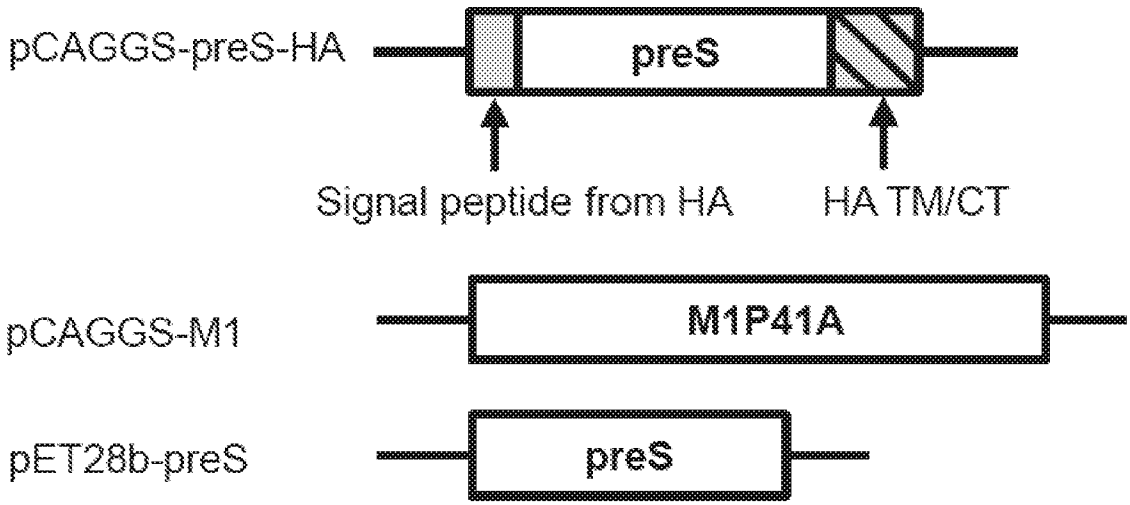

pCAGGS-preS-HA
preS
Signal peptide from HA
HA TM/CT
pCAGGS-M1
M1P41A
pET28b-preS
preS

FIG. 1A

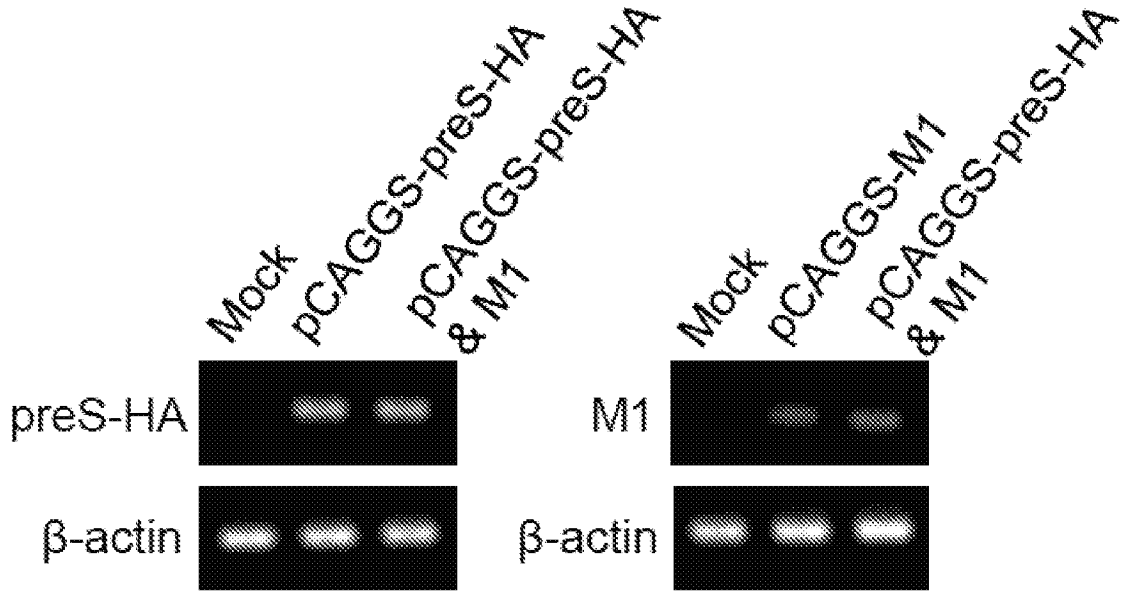

Mock
pCAGGS-preS-HA
pCAGGS-preS-HA & M1
Mock
pCAGGS-M1
pCAGGS-preS-HA & M1
preS-HA
β-actin
M1
β-actin

FIG. 1B

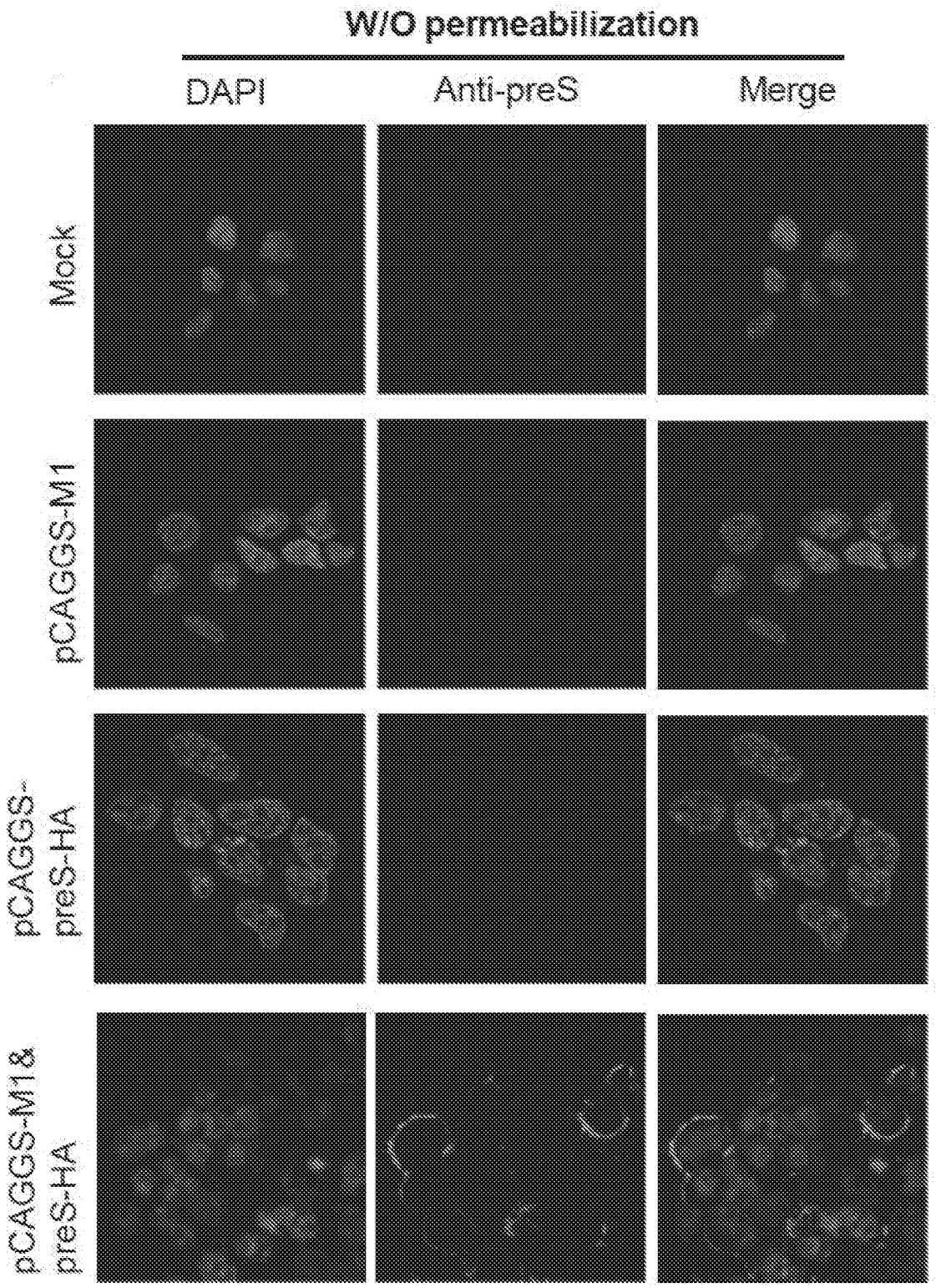

W/O permeabilization
DAPI
Anti-preS
Merge
Mock
pCAGGS-M1
pCAGGS-preS-HA
pCAGGS-M1& preS-HA

FIG. 1C

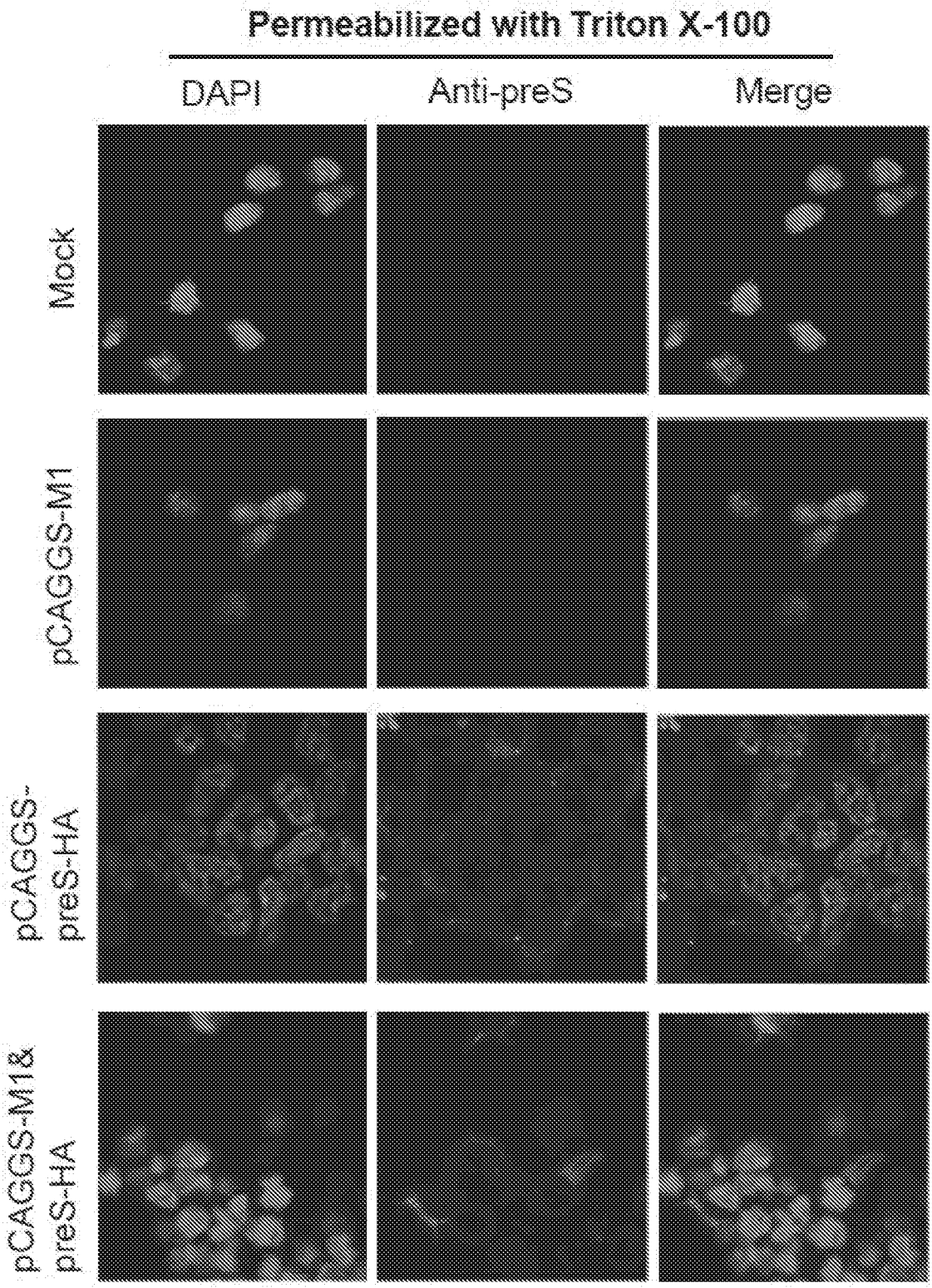

Permeabilized with Triton X-100
DAPI
Anti-preS
Merge
Mock
pCAGGS-M1
pCAGGS-preS-HA
pCAGGS-M1& preS-HA

FIG. 1D

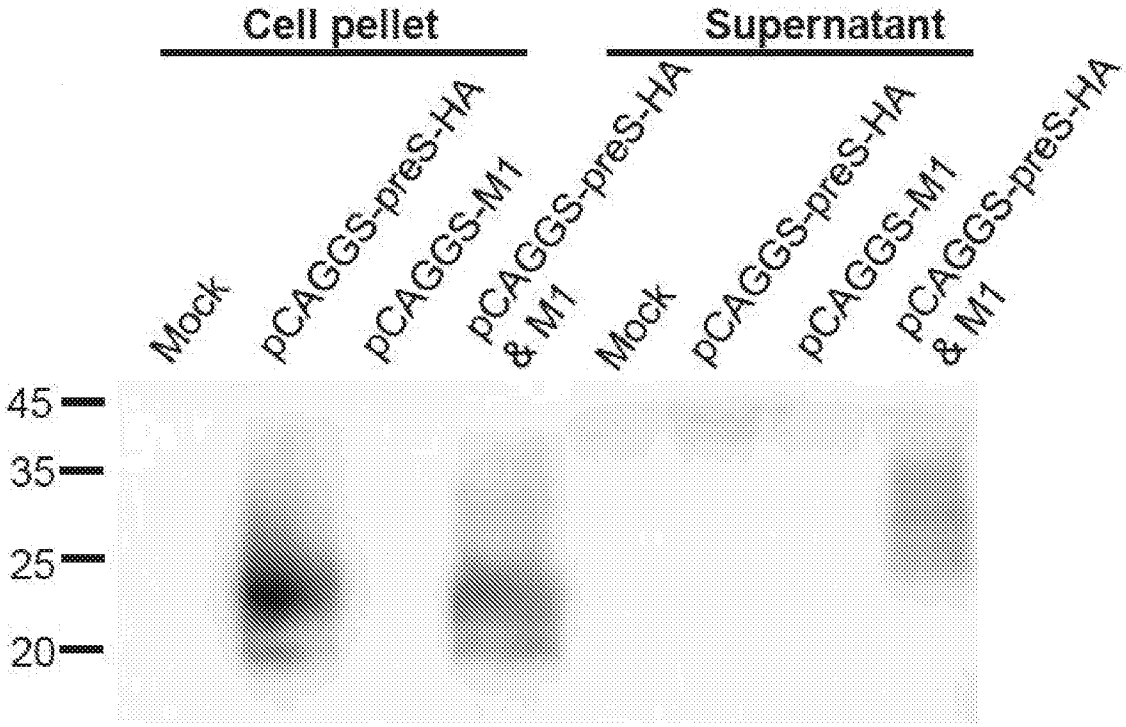


FIG. 2A

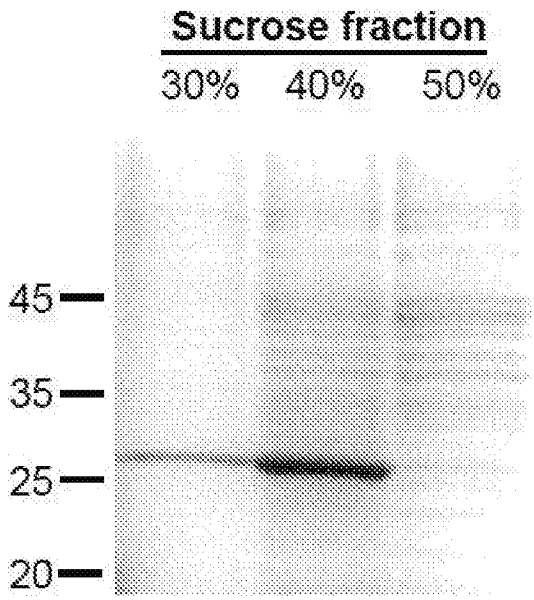


FIG. 2B

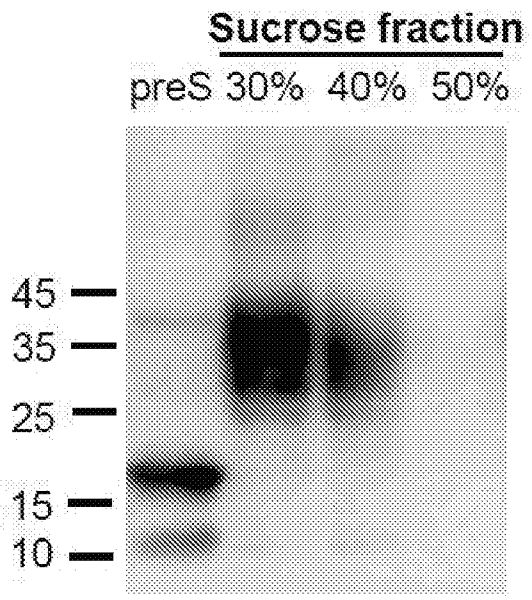


FIG. 2C

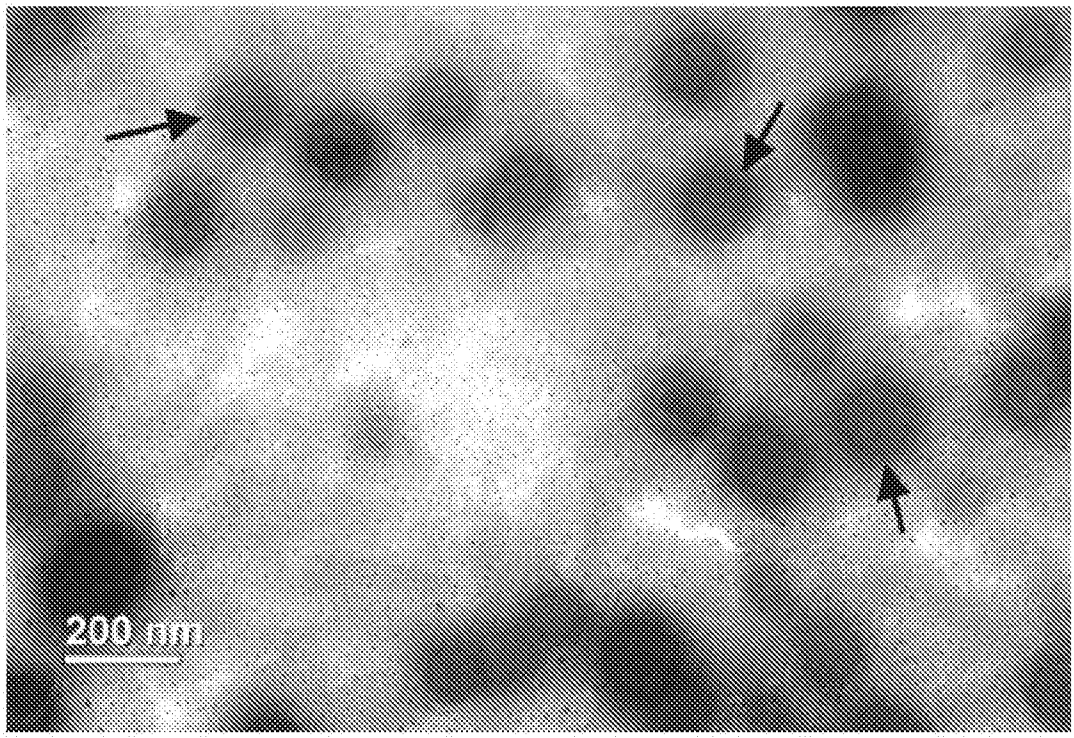


FIG. 2D

MEAKLFVLFCAFTALKAMGTNLSVPNPLGFFP
DHQLDPAFGANSNNPDWDFNPIKDHWPAANQ
VGVGAFGPGLTPPHGGILGWSPQAQGILTTVST
IPPPASTNRQSGRQPTPISPPLRDSHPQAMQW
NSTAFHQALQDPRVRGLYLPAGGSSSGTVNPA
PNIASHISSISARTGDPVTNKLESVGVHQILAIYS
TVASSLVLLVSLGAISFWMCSNGSLQCRICI

FIG. 2E

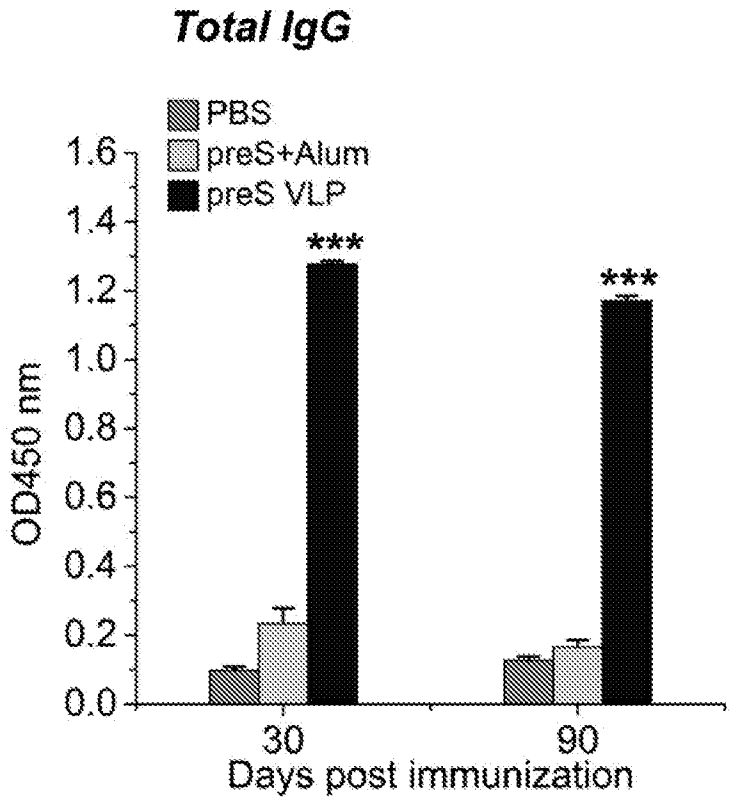

Total IgG
PBS
preS+Alum
preS VLP
OD450 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
30
90
Days post immunization

FIG. 3A

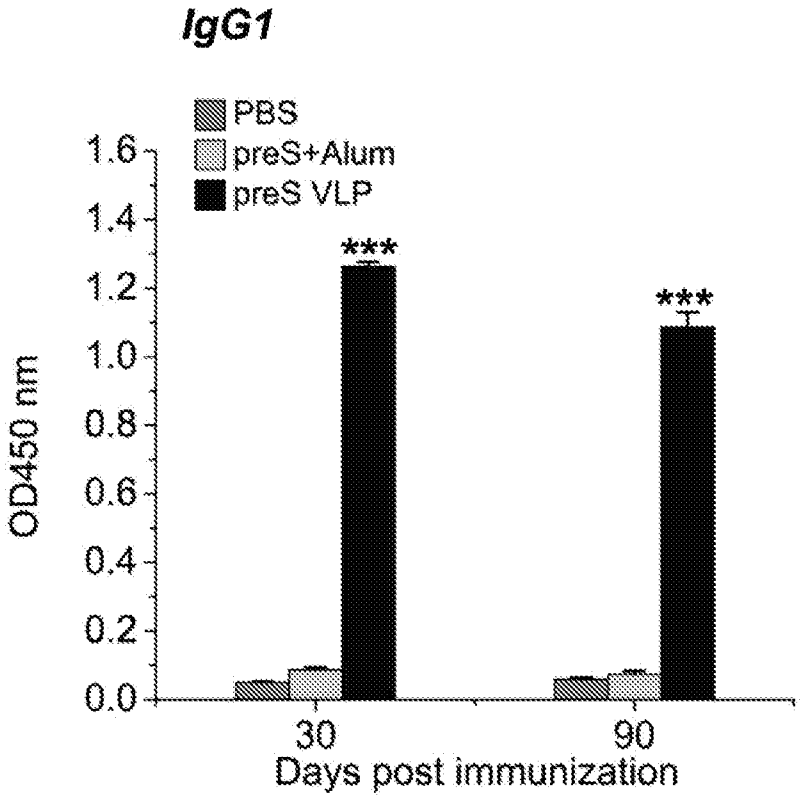

IgG1
PBS
preS+Alum
preS VLP
OD450 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
30
90
Days post immunization

FIG. 3B

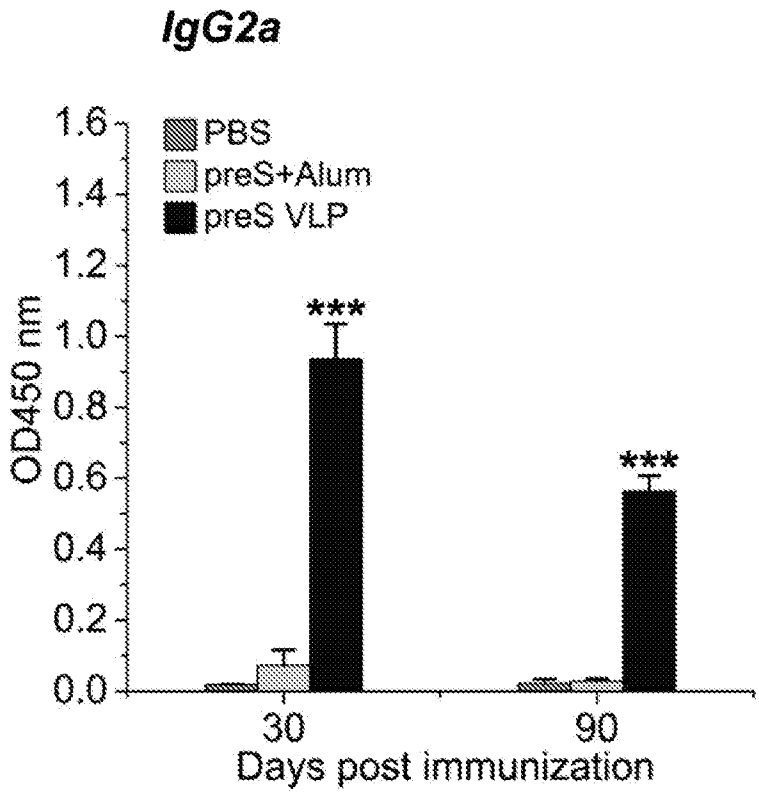

IgG2a
PBS
preS+Alum
preS VLP
OD450 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
30
90
Days post immunization

FIG. 3C

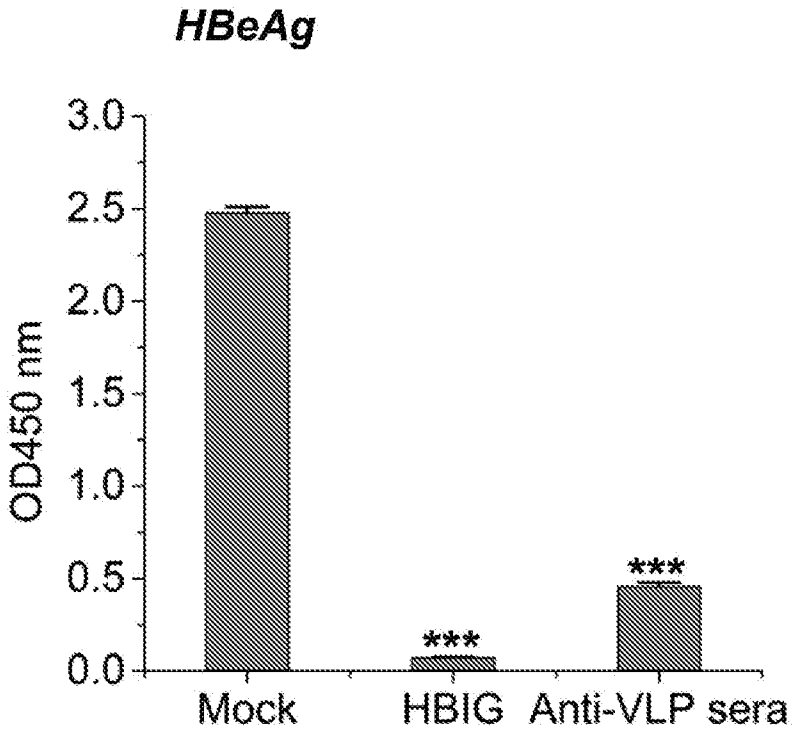

HBeAg
OD450 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
Mock
HBIG
Anti-VLP sera

FIG. 3D

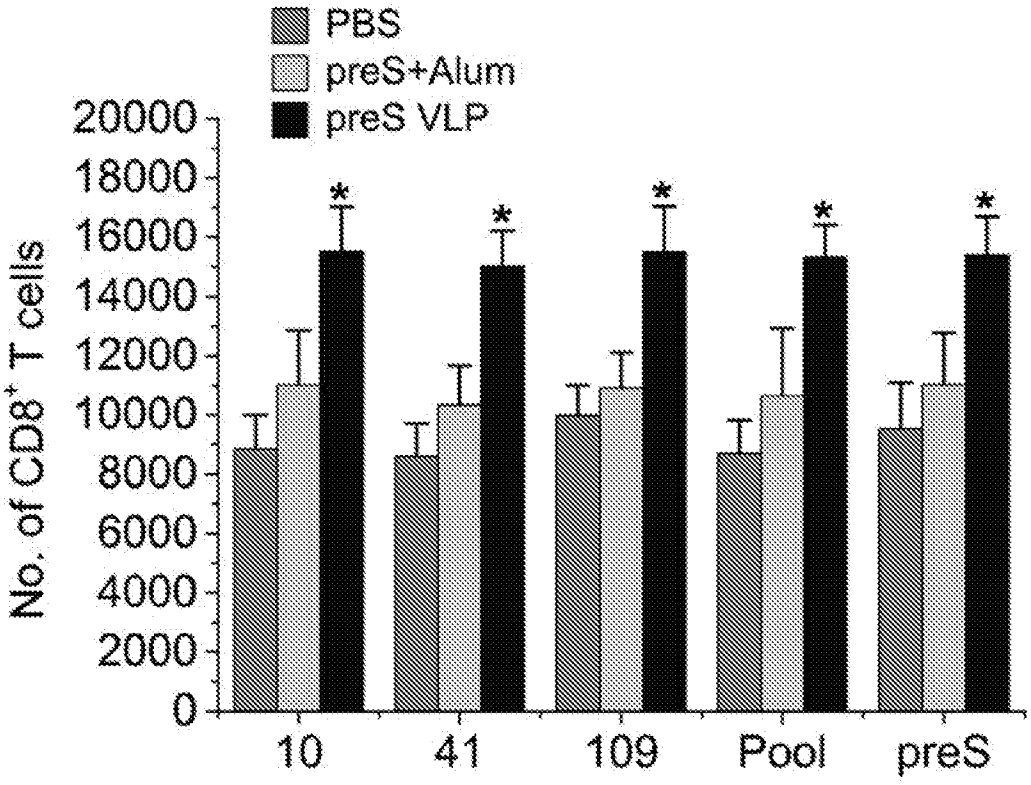

PBS
preS+Alum
preS VLP
No. of CD8+ T cells
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
10
41
109
Pool
preS

FIG. 4A

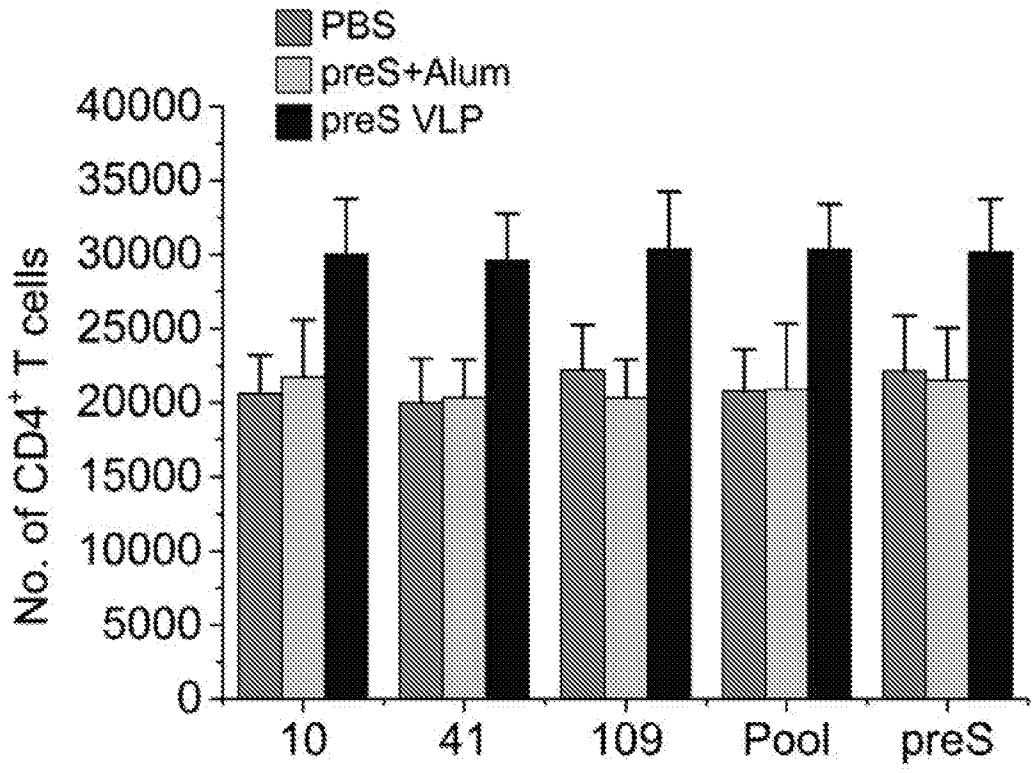

PBS
preS+Alum
preS VLP
No. of CD4+ T cells
40000
35000
30000
25000
20000
15000
10000
5000
0
10
41
109
Pool
preS

FIG. 4B

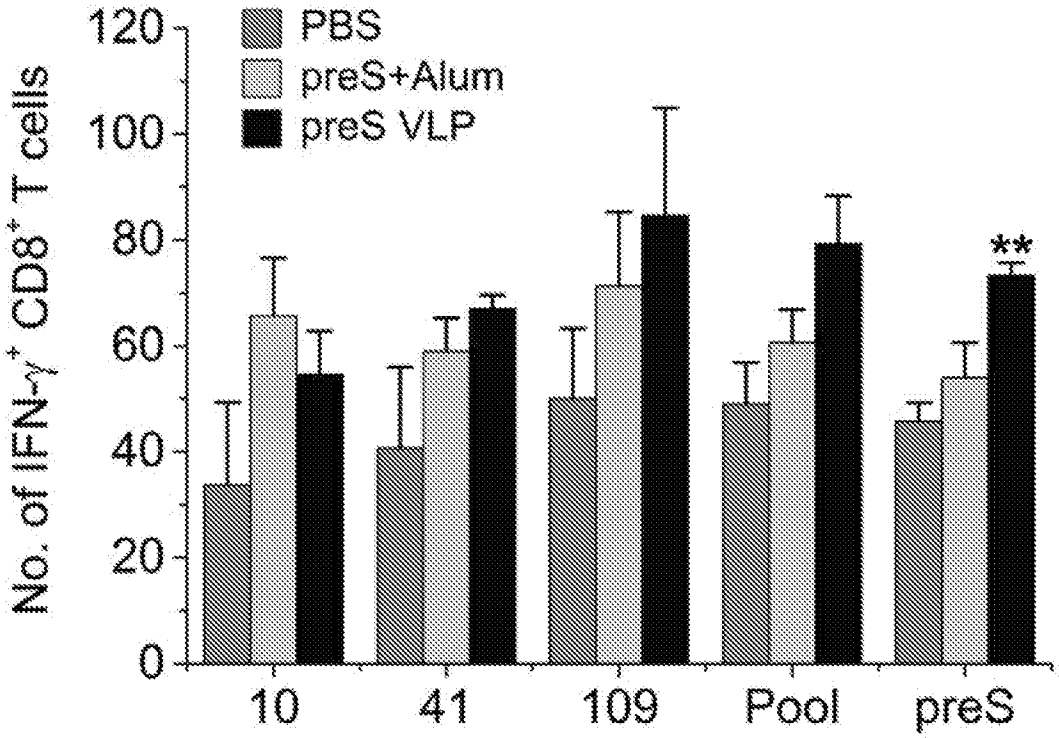

PBS
preS+Alum
preS VLP
No. of IFN-γ+ CD8+ T cells
120
100
80
60
40
20
0
**
10
41
109
Pool
preS

FIG. 4C

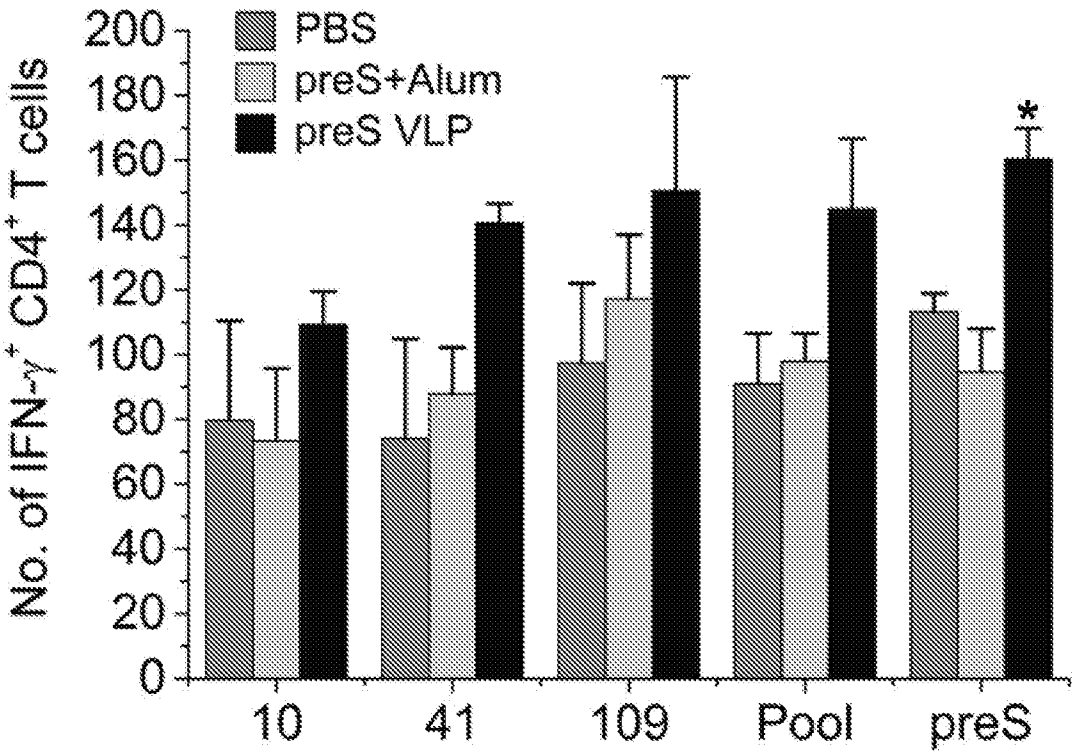

PBS
preS+Alum
preS VLP
No. of IFN-γ+ CD4+ T cells
200
180
160
140
120
100
80
60
40
20
0
*
10
41
109
Pool
preS

FIG. 4D

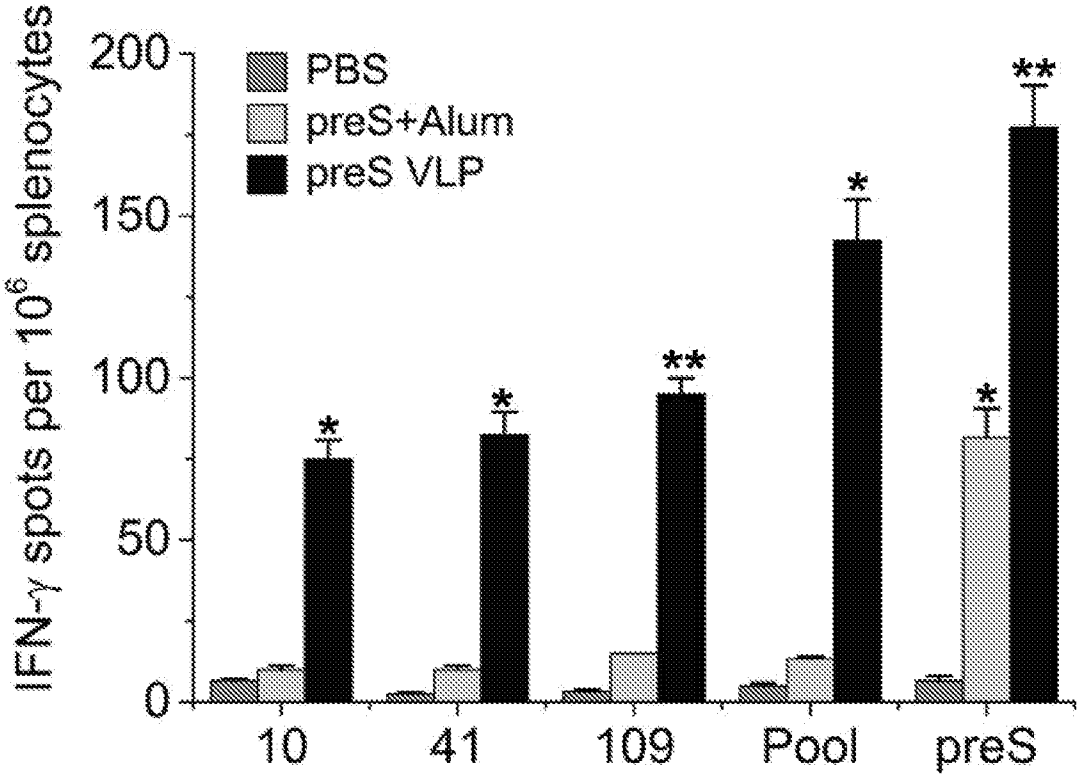

IFN-γ spots per 10^6 splenocytes
200
150
100
50
0
PBS
preS+Alum
preS VLP
*
*
**
*
*
**
10
41
109
Pool
preS

FIG. 4E

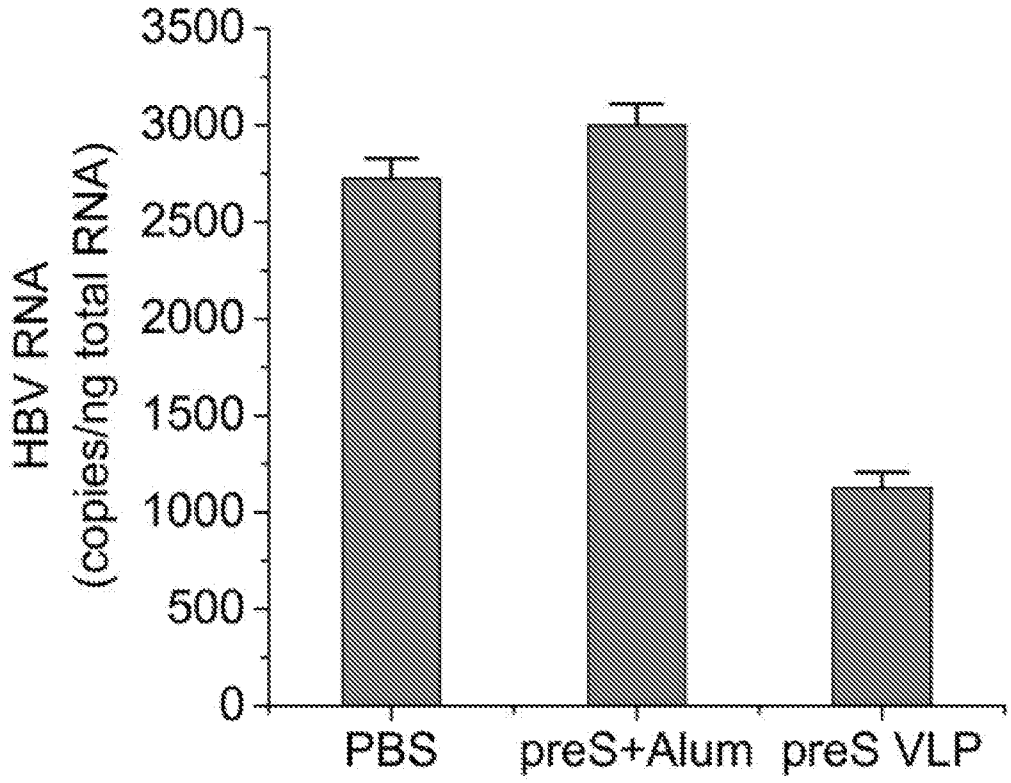

HBV RNA
(copies/ng total RNA)
3500
3000
2500
2000
1500
1000
500
0
PBS
preS+Alum
preS VLP

FIG. 5A

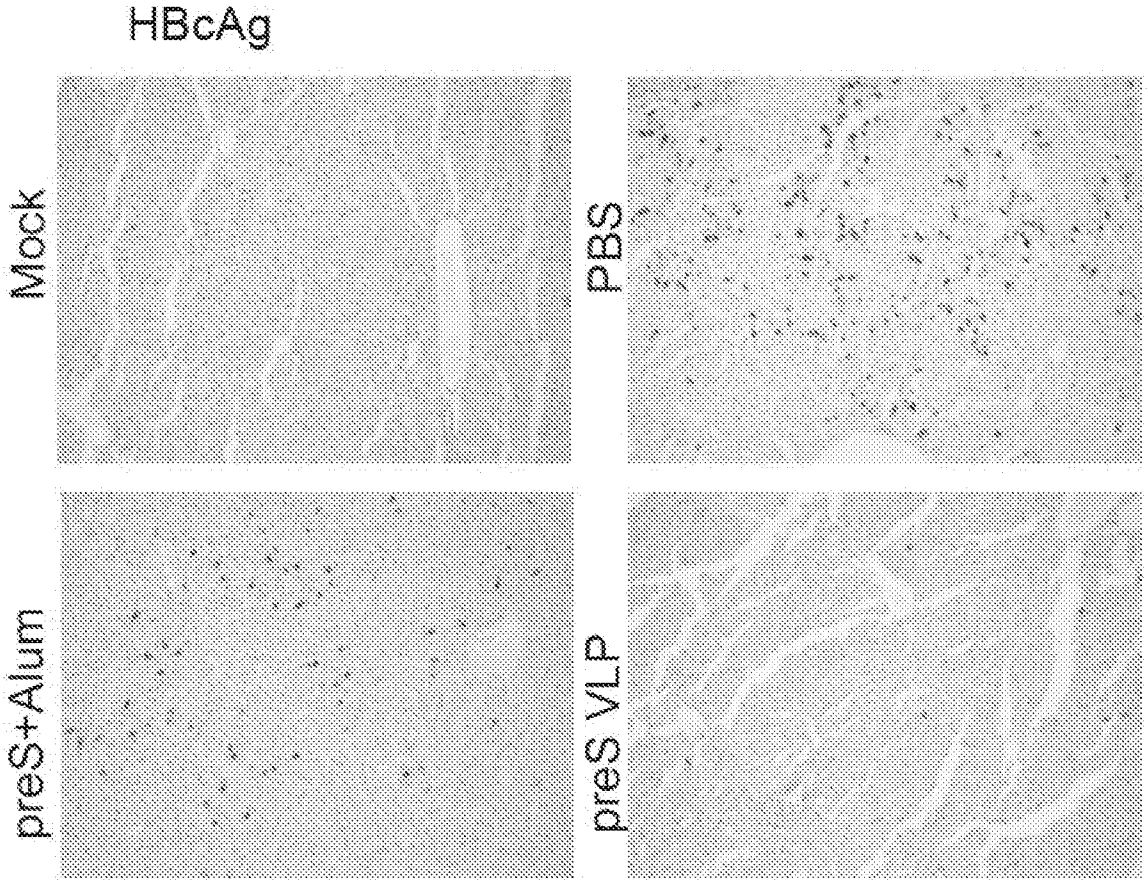


FIG. 5B

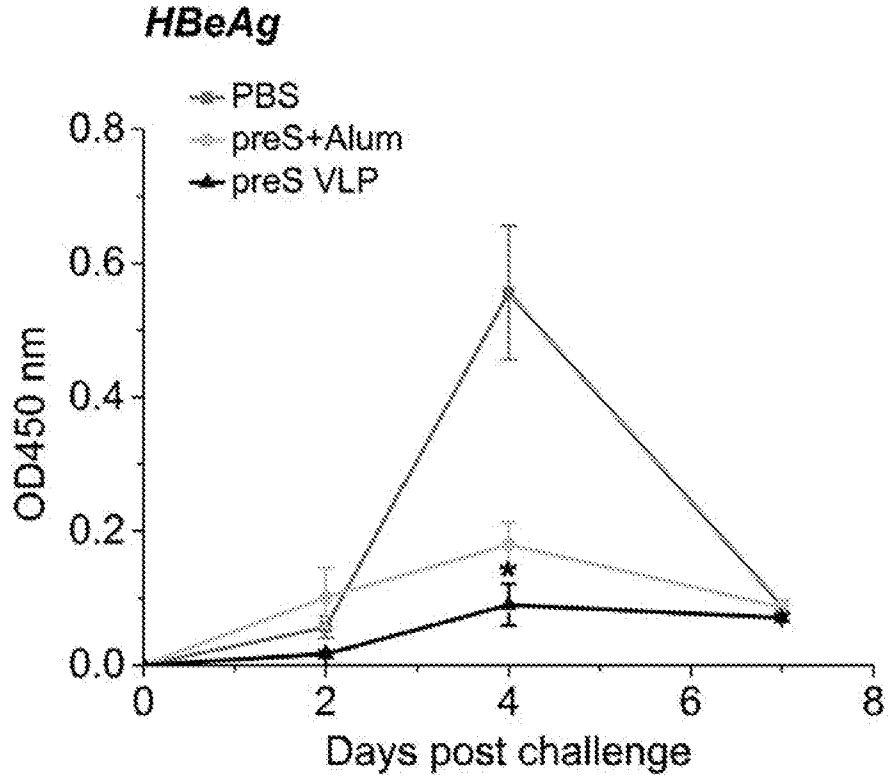

HBeAg
PBS
preS+Alum
preS VLP
0.8
0.6
0.4
0.2
0.0
OD450 nm
0
2
4
6
8
Days post challenge
*

FIG. 5C

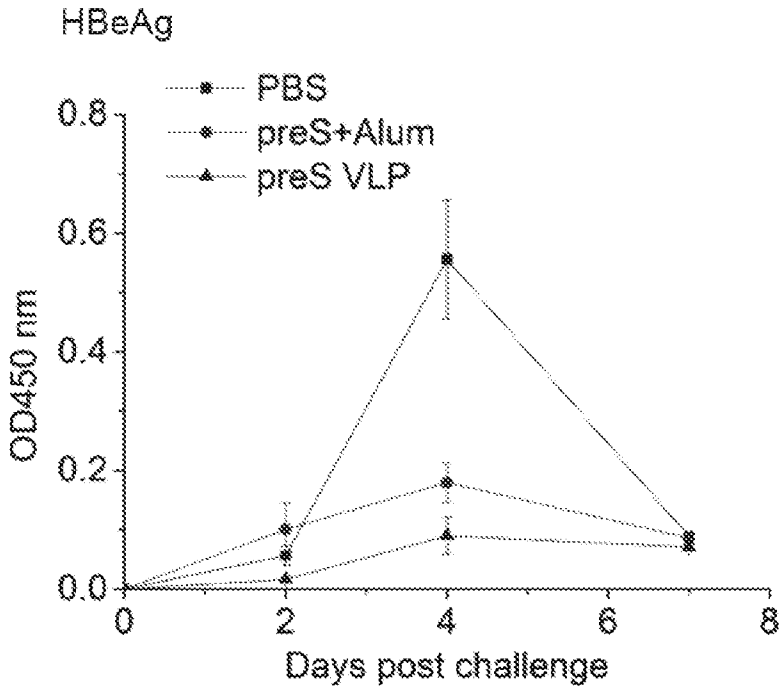

HBeAg
PBS
preS+Alum
preS VLP
0.8
0.6
0.4
0.2
0.0
OD450 nm
0
2
4
6
8
Days post challenge

FIG. 5D

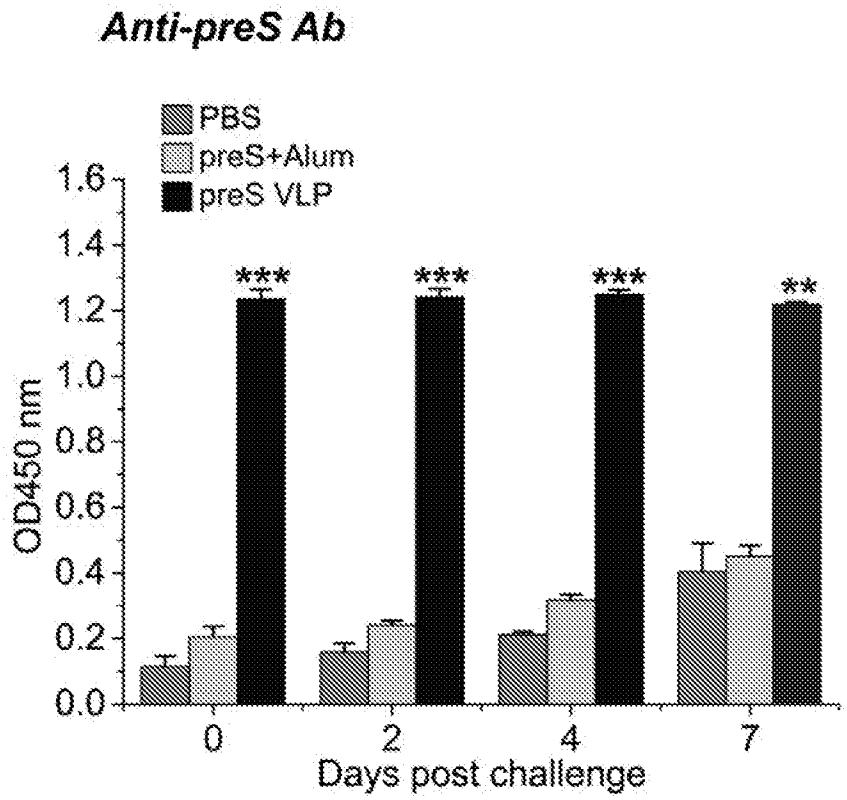

Anti-preS Ab
PBS
preS+Alum
preS VLP
OD450 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
**
0
2
4
7
Days post challenge

FIG. 5E

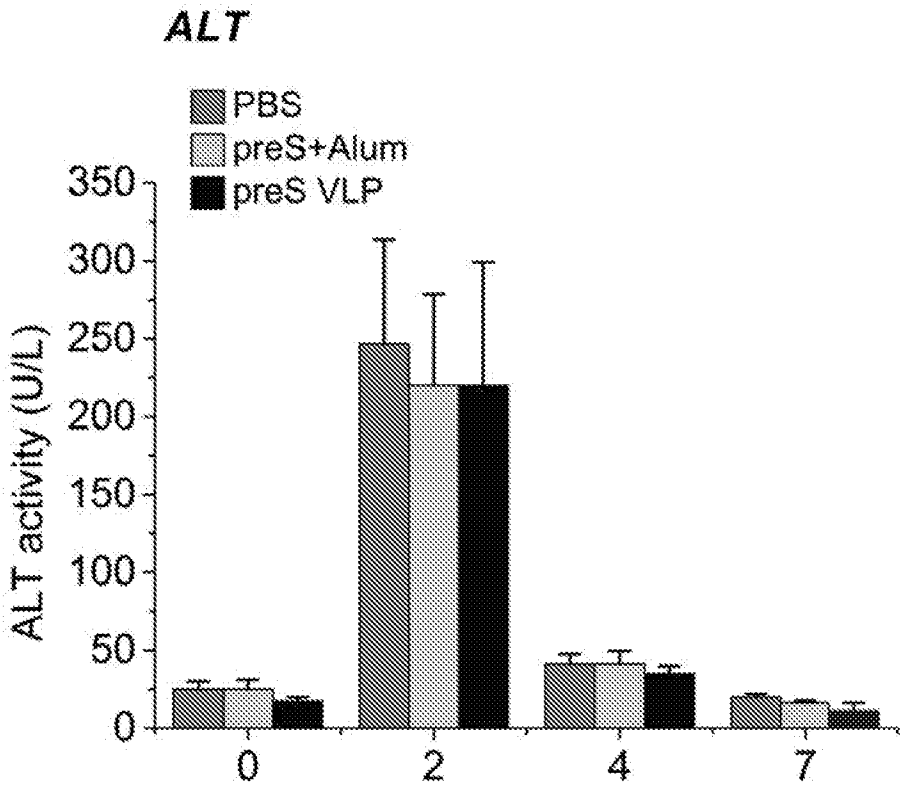

ALT
PBS
preS+Alum
preS VLP
ALT activity (U/L)
350
300
250
200
150
100
50
0
0
2
4
7

FIG. 5F

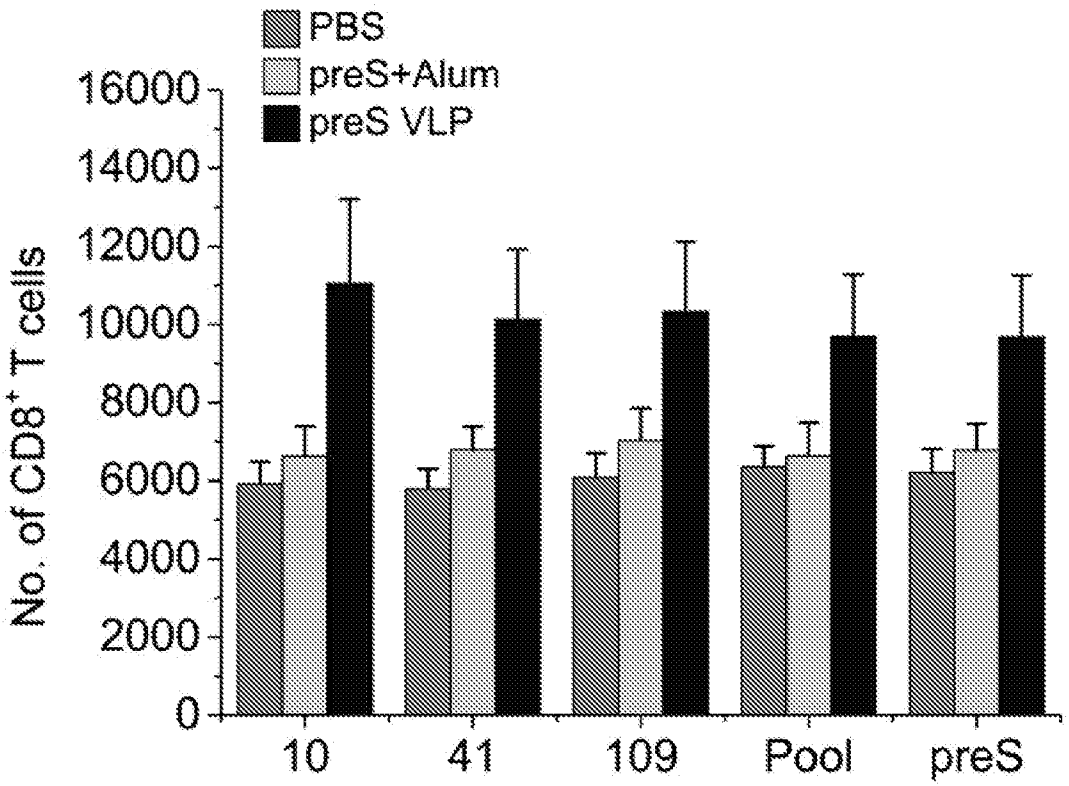

PBS
preS+Alum
preS VLP
No. of CD8+ T cells
16000
14000
12000
10000
8000
6000
4000
2000
0
10
41
109
Pool
preS

FIG. 6A

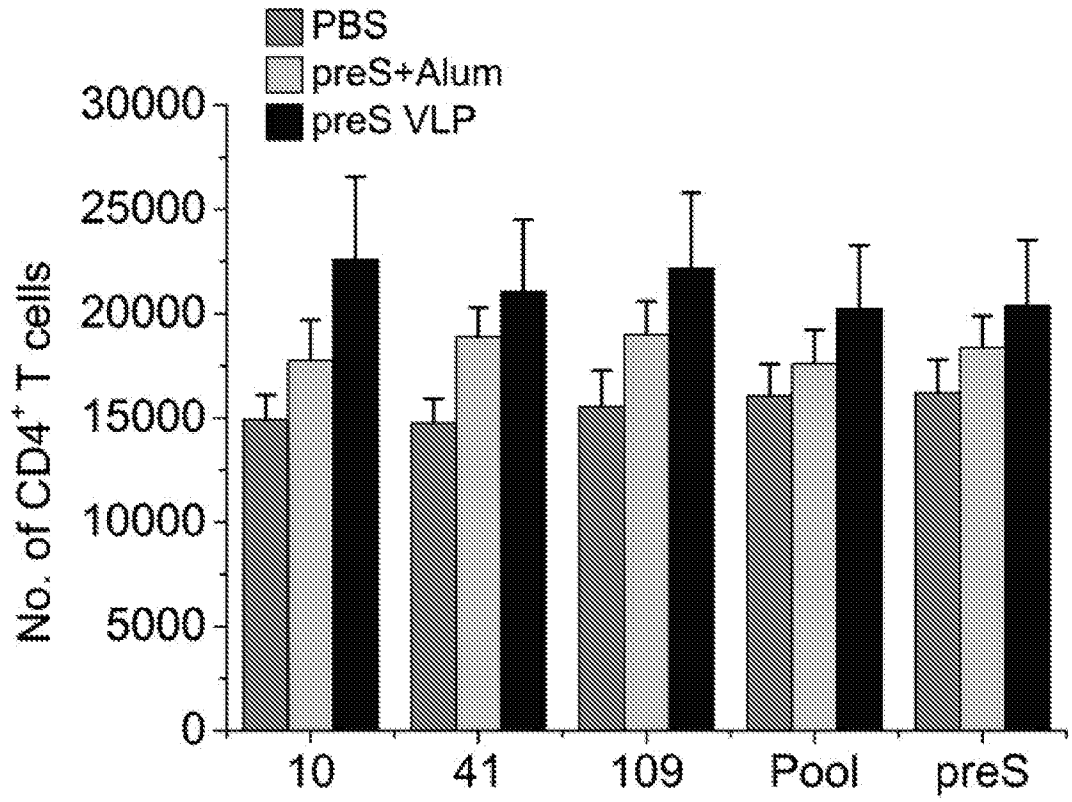

PBS
preS+Alum
preS VLP
No. of CD4+ T cells
30000
25000
20000
15000
10000
5000
0
10
41
109
Pool
preS

FIG. 6B

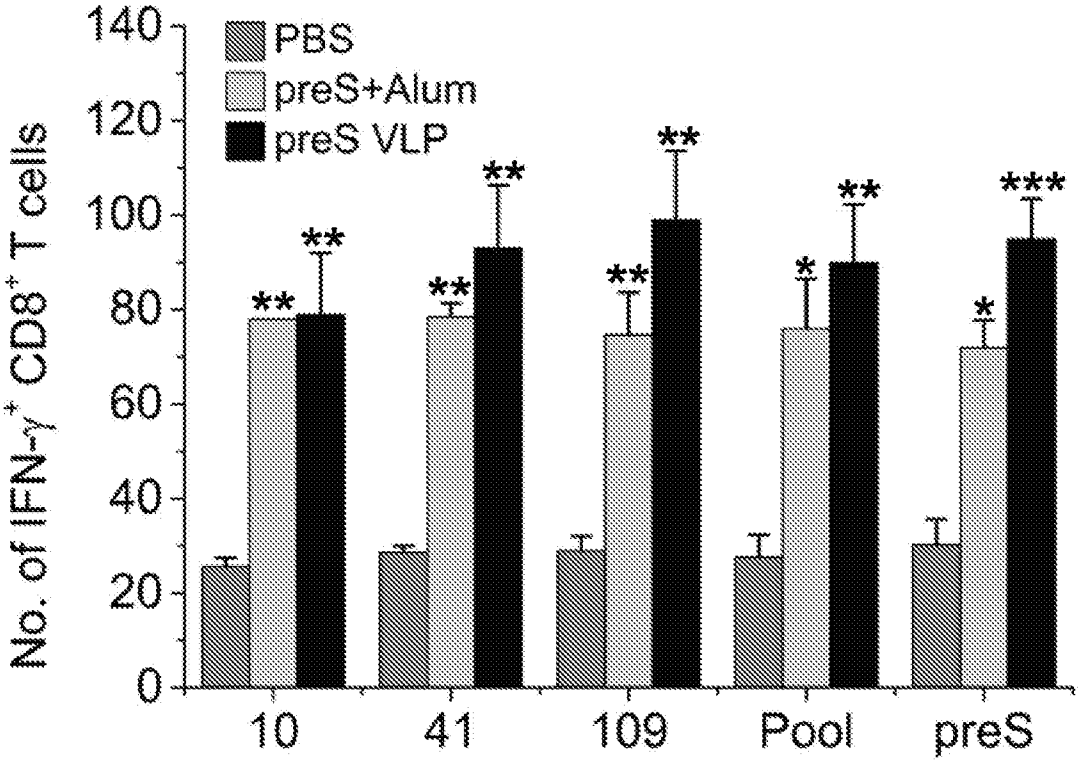

PBS
preS+Alum
preS VLP
No. of IFN-γ+ CD8+ T cells
140
120
100
80
60
40
20
0
10
41
109
Pool
preS

FIG. 6C

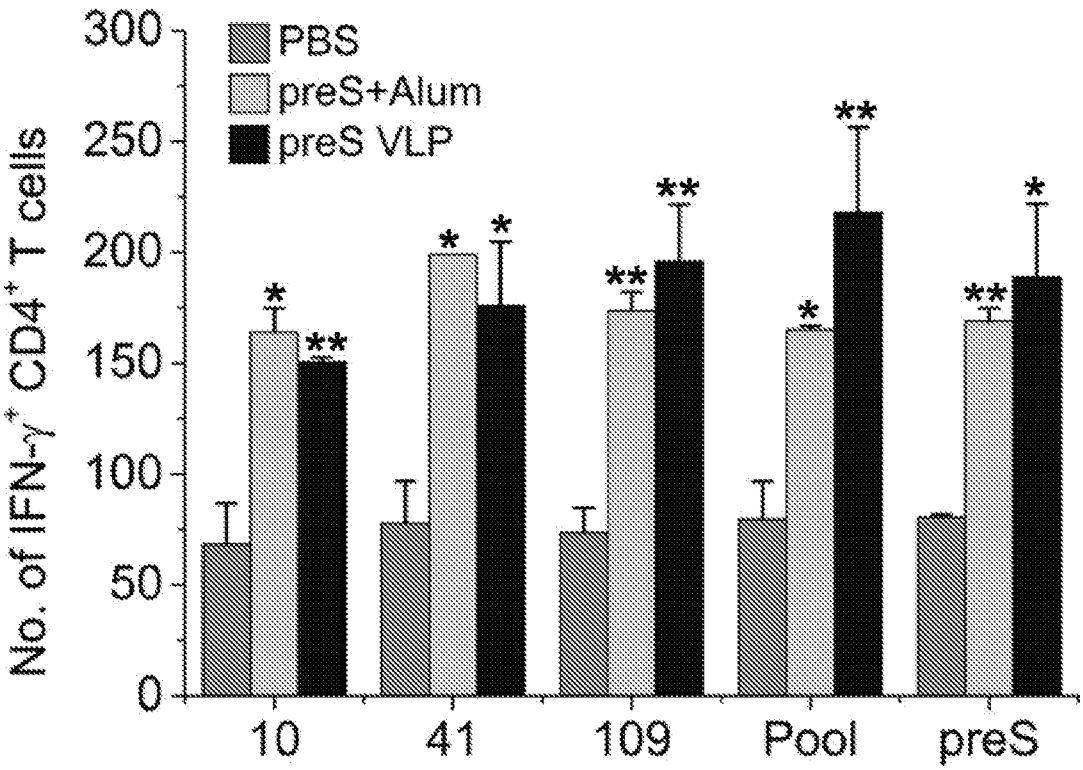

PBS
preS+Alum
preS VLP
No. of IFN-γ+ CD4+ T cells
300
250
200
150
100
50
0
10
41
109
Pool
preS

FIG. 6D

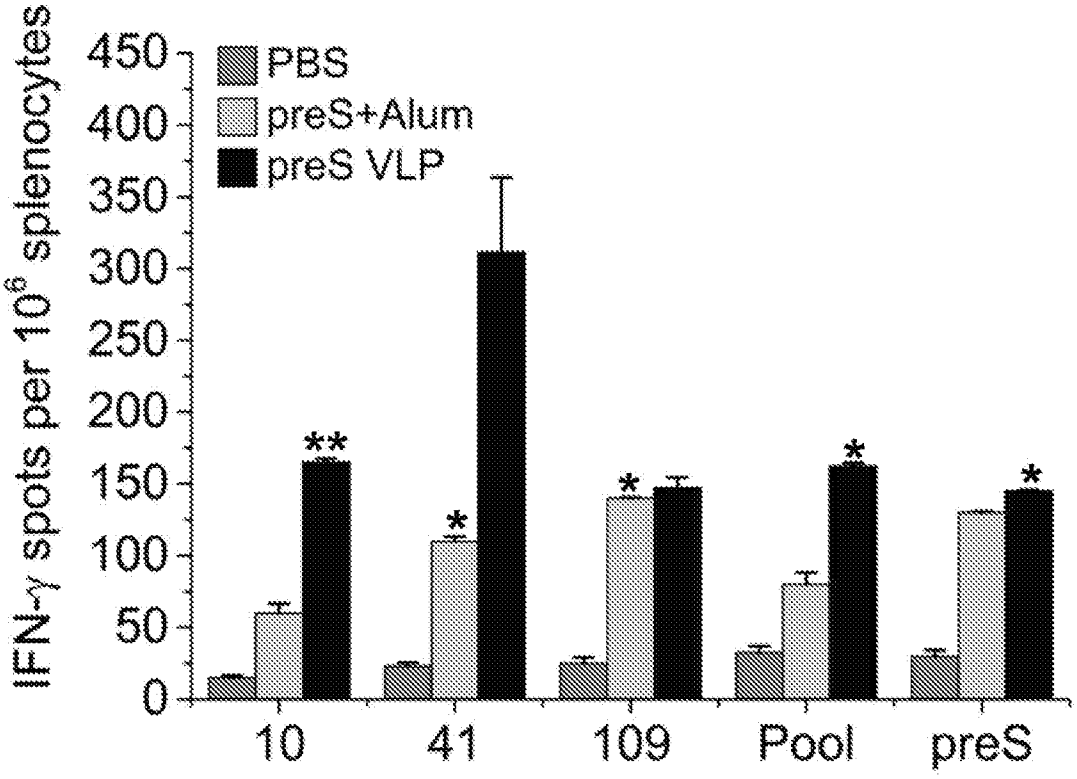

IFN-γ spots per 10^6 splenocytes
450
400
350
300
250
200
150
100
50
0
PBS
preS+Alum
preS VLP
**
*
*
*
*
10
41
109
Pool
preS

FIG. 6E

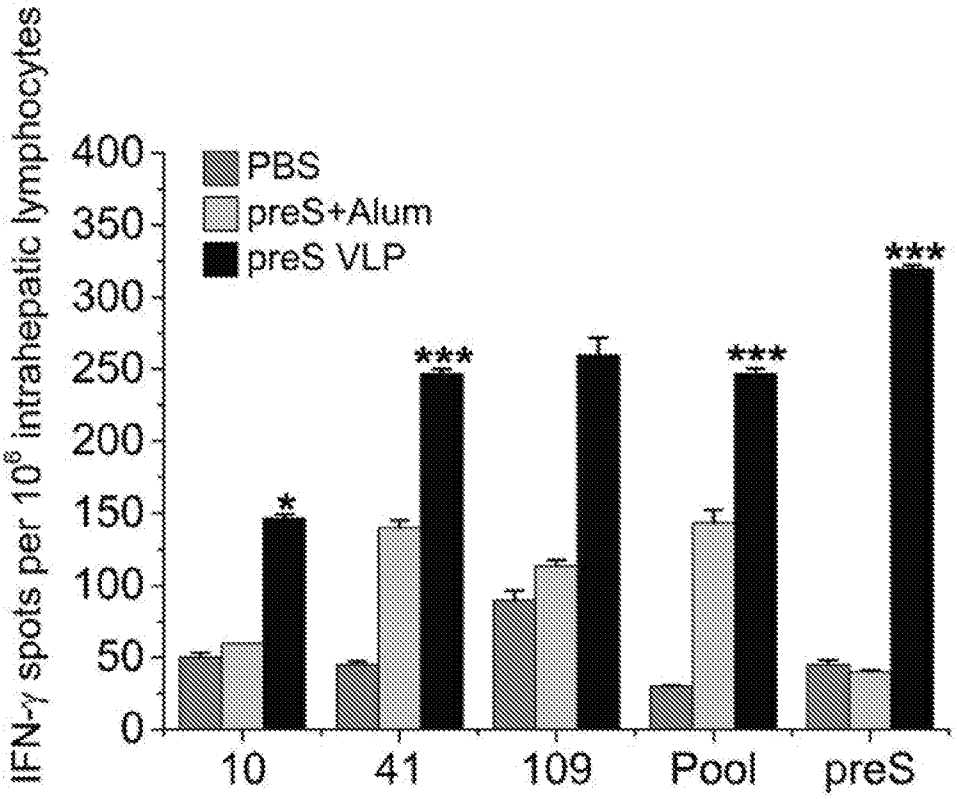

IFN-γ spots per 10^6 intrahepatic lymphocytes
400
350
300
250
200
150
100
50
0
PBS
preS+Alum
preS VLP
*
***
***
***
10
41
109
Pool
preS

FIG. 6F

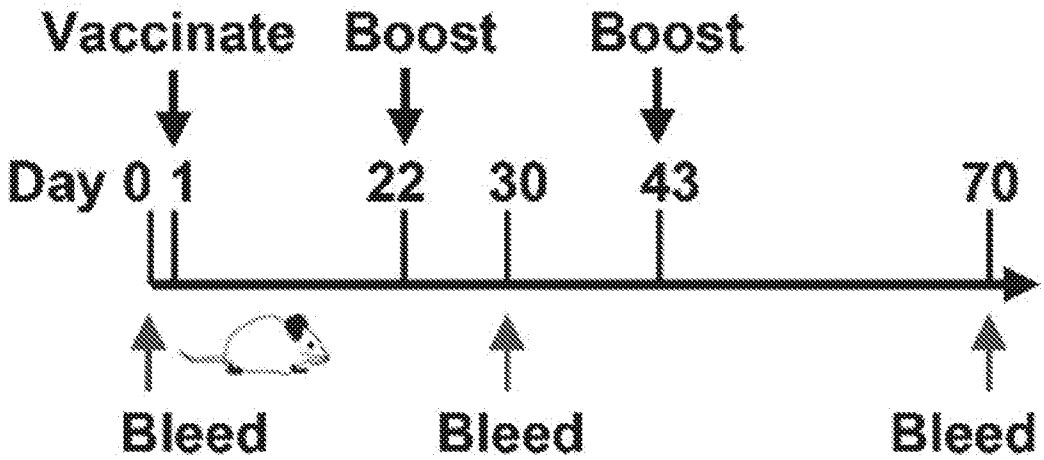


FIG. 7A

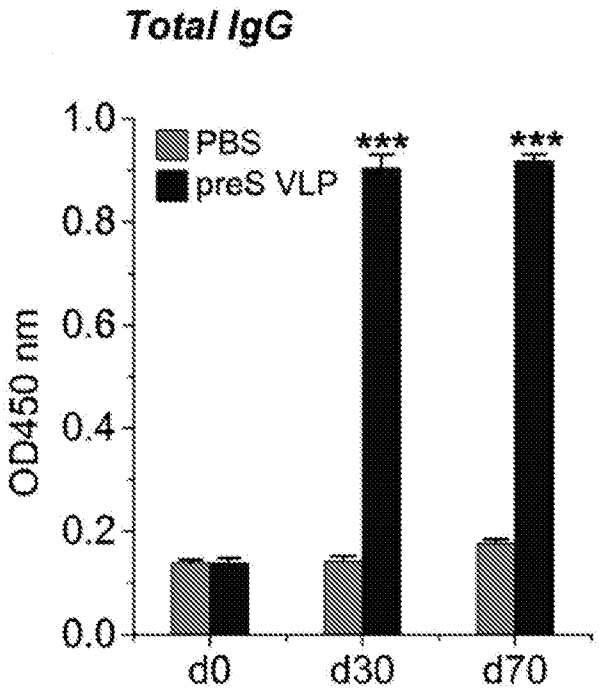


FIG. 7B

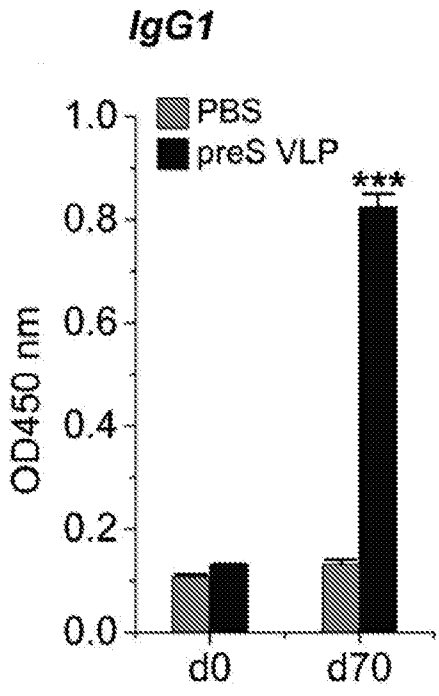


FIG. 7C

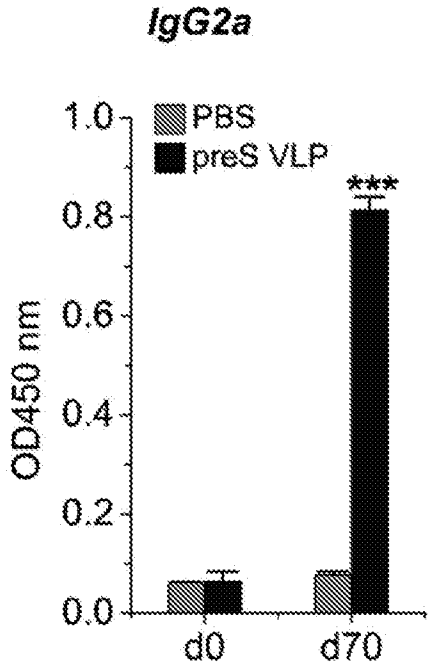


FIG. 7D

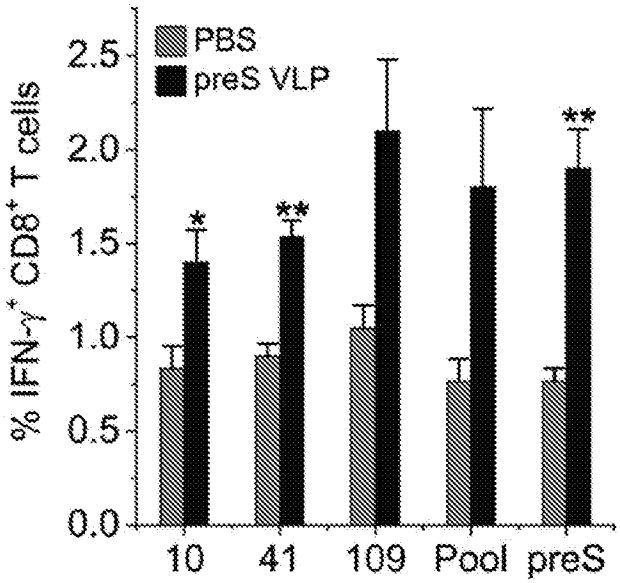


FIG. 7E

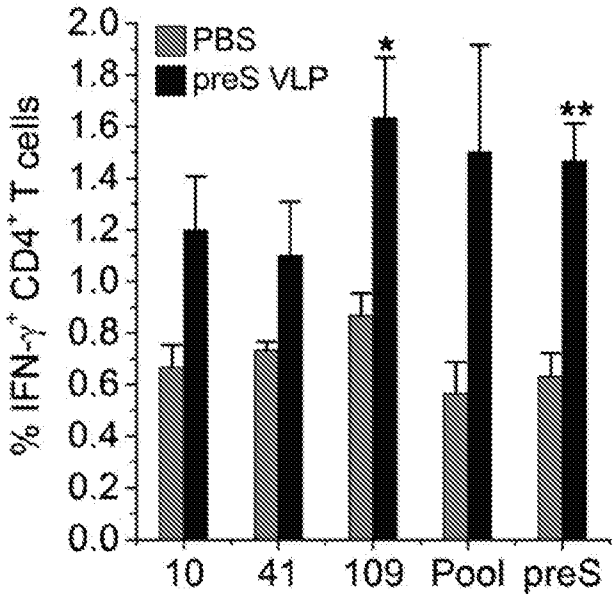


FIG. 7F

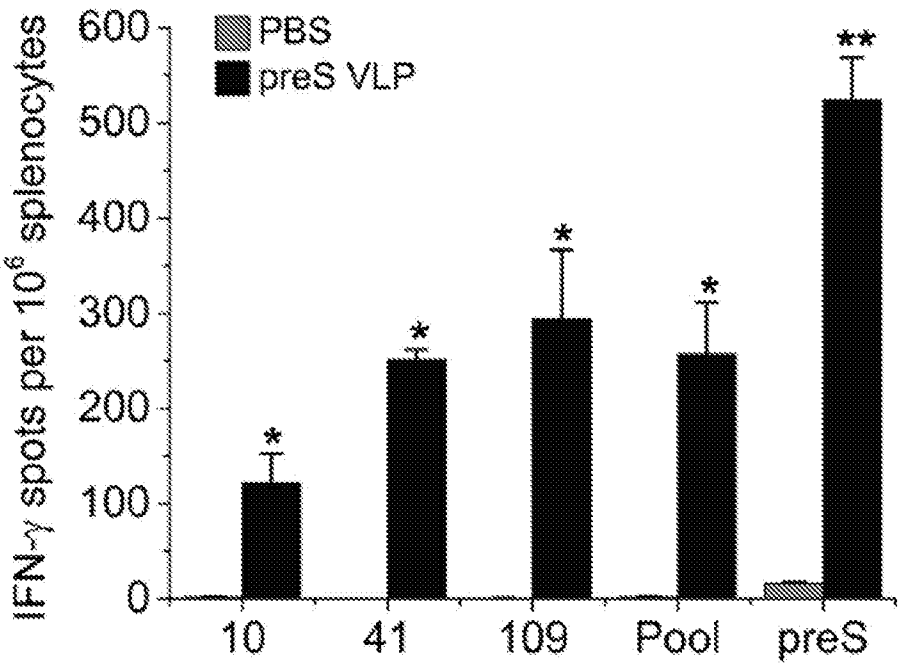

IFN-γ spots per 10^6 splenocytes
600
500
400
300
200
100
0
PBS
preS VLP
*
*
*
*
**
10
41
109
Pool
preS

FIG. 7G

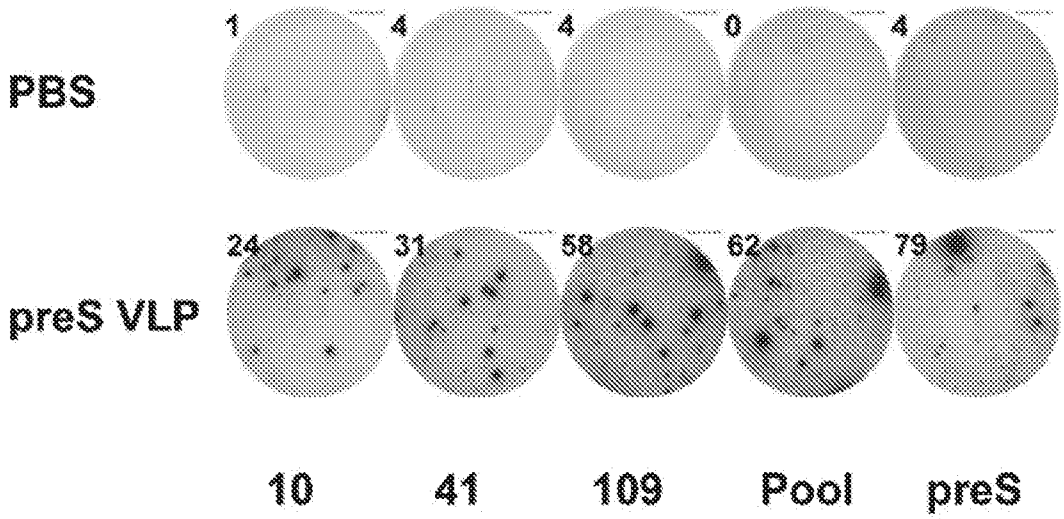

PBS
preS VLP
1
4
4
0
4
24
31
58
62
79
10
41
109
Pool
preS

FIG. 7H

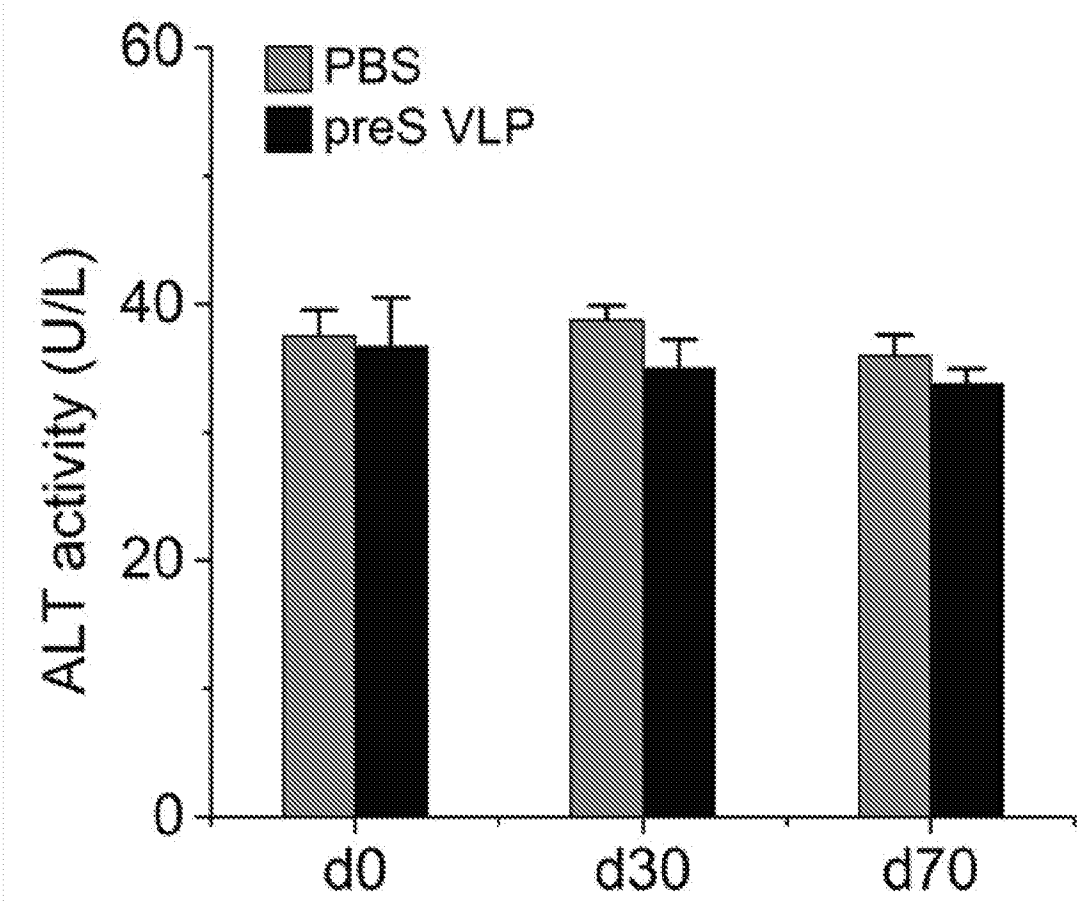


FIG. 7I

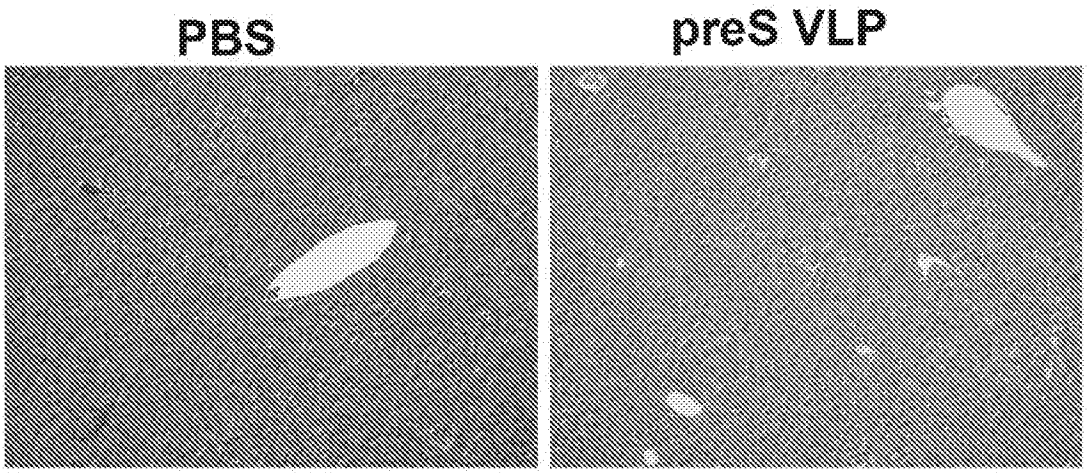


FIG. 7J

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/057390

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/00; A61K 39/00; A61K 39/29; A61P 31/12; A61P 43/00; C12N 5/10 (2017.01)

CPC - A61K 38/00; A61K 39/12; A61K 39/292; A61K 39/39; C07K 14/005; C07K 2319/00; C12N 15/85; C12N 2730/10122; C12N 2730/10134; C12N 2760/16122 (2017.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 424/185.1; 424/227.1; 435/69.1; 514/12 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2011/0257080 A1 (CHAI et al) 20 October 2011 (20.10.2011) entire document	1, 2 ------ 3, 4
Y	US 2015/273048 A1 (GEORGIA STATE UNIVERSITY RESEARCH FOUNDATION) 01 October 2015 (01.10.2015) entire document	3, 4
P, X	CN 106928372 A (PEKING UNIVERSITY SHENZHEN GRADUATE SCHOOL) 07 July 2017 (07.07.2017) entire document; see machine translation	1-5
A	WO 2006/045532 A2 (BERNA BIOTECH AG) 04 May 2006 (04.05.2006) entire document	1-5
A	WO 2012/168487 A1 (BIOMAY AG et al) 13 December 2012 (13.12.2012) entire document	1-5

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 December 2017	19 JAN 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/057390

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1 and 2 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/057390

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.