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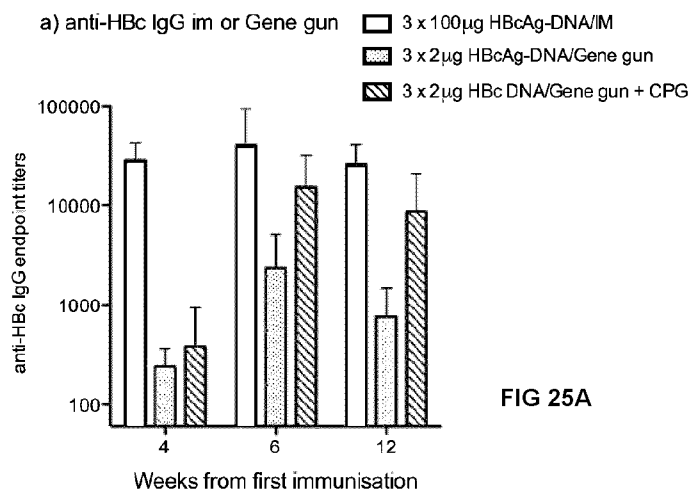


FIG 25A

(57) Abstract: Aspects of the present invention relate to chimeric polypeptides including HCV NS3/4A sequences and T-cell epitopes. Embodiments include nucleic acids encoding the chimeric NS3/4A polypeptides, the encoded polypeptides, compositions containing said nucleic acids, compositions containing said chimeric polypeptides, as well as methods of making and using the aforementioned compositions including, but not limited to medicaments and vaccines.

IMMUNOGEN PLATFORM

REFERENCE TO SEQUENCE LISTING

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled TRIPEP112VPC.TXT, created February 16, 2009, which is 4.8 MB in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This international application claims the benefit of priority to PCT Application No. PCT/IB2008/003047, filed August 15, 2008, which was written in English and designated the United States of America and this application claims the benefit of priority to U.S. Provisional Application No. 61/047,076, filed April 22, 2008, and U.S. Provisional Application No. 61/149,299, filed February 2, 2009.

BACKGROUND

[0003] Traditionally, vaccines have been based on live attenuated or inactivated pathogens. These strategies are inefficient, however, largely because of the antigenic variability of pathogens (e.g., viruses). Several peptide vaccines that comprise antigenic peptides or peptide fragments of pathogens have been developed. Conserved peptide fragments are less likely to exhibit antigenic variability and can overcome some of the problems associated with traditional peptides. Accordingly, subunit vaccines have been developed, which target conserved regions of pathogens. Synthetic peptide vaccines tend to be poorly immunogenic, however. The poor immunogenicity of synthetic peptide vaccines may be attributed to the fact that although these types of vaccines induce humoral antibody responses, they are less likely to induce cell-mediated responses.

[0004] Several investigators have sought to improve the antigenicity of synthetic peptide vaccines. For example, Klein et al. describe the engineering of chimeric proteins that comprise an immunogenic region of a protein from a first antigen linked to an immunogenic region from a second pathogen. (See, U.S. Pat. Nos. 6,033,668; 6,017,539; 5,998,169; and 5,968,776). Others have sought to create chimeric proteins that couple B-cell epitopes to

universal T-cell epitopes in order to improve the immune response. (See, e.g., U.S. Pat. No. 5,114,713). Russell-Jones et al. (U.S. Pat. No. 5,928,644) also disclose T-cell epitopes derived from the TraT protein of *Escherichia coli*, which are used to produce hybrid molecules so as to generate an immune response to parasites, soluble factors (e.g., LSH) and viruses. Further, Ruslan (U.S. Patent Application Publication No. 20030232055) discloses the manufacture of vaccines based on PAMPs and immunogenic antigens. Despite these advances, the development of compositions and methods that improve the antigenicity of immunogens is manifest.

[0005] The hepatitis B virus core antigen (HBcAg) is thought to be a key target for the host immune response in the control of the infection. In particular, the presence of HBcAg-specific T cells has been associated with clearance of acute and chronic infections with the hepatitis B virus (HBV). Subsequently, prophylactic and therapeutic vaccines that induce HBcAg-specific T cells have been developed and have shown some efficiency in infectious models. However, despite the high immunogenicity of exogenous HBcAg many of the studies using endogenous HBcAg as a vaccine have been disappointing. The reason for this is not fully understood.

[0006] When expressed alone, HBcAg will spontaneously assemble into virus-like particles (VLPs) that are highly immunogenic *in vivo*. These VLPs use B cells as the primary antigen-presenting cell (APC) by an unusual interaction with the B cell receptor. HBcAg effectively prime specific T helper (Th) and, much less effectively, cytotoxic T cells (CTLs) as an exogenous antigen when high antigen doses in adjuvant are used. Both DNA- and retrovirus-based immunizations using HBcAg have been reported to induce detectable HBcAg-specific CTLs in mice. One embodiment presented herein is the intrinsic immunogenicity of HBcAg as an endogenous antigen.

[0007] DNA vaccines can be used as a model to study the endogenous immunogenicity of antigens. However, a number of phase I/II clinical trials have now shown that direct intramuscular injections of DNA vaccines fails to prime robust immune responses in humans. Thus, DNA-based immunizations should be evaluated by approaches that are immunogenic in humans. Different modes of DNA delivery that can be used in humans have now become available, including transdermal delivery of DNA coated to gold beads using the gene gun or treatment of the injection site by *in vivo* electroporation.

SUMMARY OF THE INVENTION

[0008] Several embodiments described herein concern compositions and methods that are useful for the generation, enhancement, or improvement of an immune response to a target antigen. Many platforms for the presentation of antigens are provided. These platforms are particularly useful for nucleic acid-based immunogens (e.g., DNA vaccines). It has been discovered that the hepatitis C virus (HCV) nonstructural protein 3 (NS3) and nonstructural protein 4A (NS4A), collectively NS3/4A, and fragments of this fusion protein (e.g., fragments that retain the protease domain, protease cleavage site, and/or the helicase domain) or a nucleic acid encoding these proteins are useful platforms to present antigens (e.g., nucleic acids encoding a T cell epitope, such as a CTL or HTL domain) so as to generate a potent immune response to the associated antigen.

[0009] One aim of using NS3/4A as a carrier or adjuvant is to effectively provide T helper cells access to a fused antigen, thereby enhancing the immune response to the fused antigen. In addition, in some embodiments it is desired to have an active, or highly active, NS3/4A protease since an enhanced or altered protease activity can have adjuvant effects that improve the immune response to the fused gene. Moreover, the active NS3/4A protease can be used to cleave the fused protein (e.g., a heterologous antigen), especially when it contains inserted heterologous protease cleavage sites, into smaller fragments to enhance processing and to ensure that the fused protein will not resemble its native structure. For certain conditions or diseases it can be desirable to use the fused protein in a way that is structurally different from the native form since the native form of the protein may have properties that are at an immunogenic disadvantage. It is envisioned that the introduction of foreign protease cleavage sites in the fusion protein (e.g., a peptide antigen) induce protein cleavage into small fragments that can enhance processing. Additionally, if the natural sequence has been changed the cleavage at the introduced sites can ensure that no new, artificial junctional T cell epitopes are generated.

[0010] Accordingly, embodiments disclosed herein include compositions that comprise an isolated nucleic acid that encodes a chimeric Hepatitis C virus (HCV) NS3/4A polypeptide or a fragment thereof, which comprises a sequence that encodes an antigen

(preferably a non-HCV epitope or, when an HCV epitope is used, the antigen is not in a position on the NS3/4A peptide or NS3/4A nucleic acid or fragment thereof that is naturally occurring). The immunogenic sequence (e.g., a nucleic acid encoding an antigen) can be inserted within the NS3/4A nucleic acid or NS3/4A peptide or attached thereto (e.g., the antigen-encoding nucleic acid can be inserted within the nucleic acid encoding NS3 and/or NS4A domain at a location outside of or within the protease domain, helicase domain, or protease cleavage site or said sequence encoding said antigen can be flanking the 5' or 3' end of the nucleic acid encoding said HCV NS3/4A peptide, such as juxtaposed to the 5' or 3' end).

[0011] Embodiments also include an antigenic or immunogenic peptide (e.g., a peptide of a pathogen, bacteria, toxin, virus, or cancer cell antigen) joined to (e.g., flanking or juxtaposed to) or affixed within NS3/4A peptide or a fragment thereof, which peptide fragment can comprise at least, equal to, greater than, less than, or any number in between 3, 5, 10, 20, 50, 100, 150, 200, 250, 300, 350, 400, 500, 700, 1000, 1200, or 1500 consecutive amino acids of a natural or synthetic NS3/4A polypeptide (e.g., a naturally occurring isotype of NS3/4A or a codon-optimized or otherwise modified NS3/4A polypeptide (e.g., consensus NS3/4A sequences generated from 2 or more istotypes), such as **SEQ. ID. Nos. 2 and 36**).

[0012] More embodiments include a nucleic acid that encodes an antigenic or immunogenic peptide (e.g., a nucleic acid encoding a peptide of a pathogen, bacteria, toxin, virus, or cancer cell antigen) joined to or affixed within a nucleic acid encoding an NS3/4A peptide or a fragment thereof (e.g., the nucleic acid that encodes said antigenic or immunogenic peptide can be inserted within or joined to (e.g., flanking or juxtaposed to) an isolated but naturally occurring NS3/4A nucleic acid or a synthetic NS3/4A nucleic acid (e.g., a codon-optimized NS3/4A nucleic acid) or a fragment of these nucleic acids (e.g., the NS3/4A nucleic acid fragment can comprise, consist of, or consist essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, or 2500 consecutive nucleotides of a nucleic acid sequence that encodes an isolated but natural NS3/4A polypeptide or synthetic NS3/4A polypeptide (e.g., **SEQ. ID. Nos. 1 and 35**)). Polypeptides encoded by said nucleic acids are also embodiments.

[0013] In some embodiments, the antigenic or immunogenic peptide is a T cell epitope (TCE), such as a CTL epitope or an HTL epitope, or the antigenic or immunogenic nucleic acid encodes a TCE, such as a CTL epitope or HTL epitope. As above, the TCE can be inserted within or flanking (e.g., juxtaposed to) the NS3/4A peptide or nucleic acids encoding the NS3/4A peptide or fragments of these peptides and nucleic acids, as described above, such that said chimeric sequences are or encode chimeric NS3/4A polypeptides or fragments thereof with TCEs inserted within or flanking (e.g., juxtaposed to) the NS3/4A polypeptide sequences. Preferably, the nucleic acid encoding the TCE, and the encoded TCE, is located at a position that is not naturally occurring in HCV, when said TCE is an HCV epitope. Desirably, the encoded NS3/4A chimeric polypeptide retains catalytic activity (e.g., protease and/or helicase activity).

[0014] Accordingly, several embodiments include a nucleic acid that encodes a TCE (e.g., a CTL or HTL of a pathogen, bacteria, virus, toxin, or of a cancer cell) inserted within or flanking (e.g., juxtaposed to) a nucleic acid encoding an NS3/4A polypeptide (e.g., **SEQ. ID. Nos. 1 or 35**) or a nucleic acid encoding a fragment of an NS3/4A polypeptide (e.g., a fragment of **SEQ. ID. Nos. 1 or 35**), preferably a fragment that retains protease and/or helicase activity, wherein said fragment can comprise, consist of, or consist essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, or 2500 consecutive nucleotides of a nucleic acid sequence that encodes an isolated but natural NS3/4A polypeptide or synthetic NS3/4A polypeptide (e.g., **SEQ. ID. Nos. 1 or 35**), such that when said TCE is a sequence that encodes an HCV TCE, said HCV TCE is in a position on the nucleic acid or polypeptide encoded by said nucleic acid that is not naturally occurring. Polypeptides encoding said nucleic acid embodiments are also aspects of the invention.

[0015] Optionally, the isolated nucleic acids can also encode a linker sequence and/or a sequence that promotes adjuvant activity (e.g., a stimulatory TCE, a plurality of immune stimulatory di nucleotides, such as CpG, or an RNA binding domain). For example, in some embodiments, the linker sequence or adjuvant sequence flanks (e.g., juxtaposed to) at least one end of the encoded TCE or antigen. Preferably, the nucleic acid encodes a linker comprising one to six alanine and/or glycine residues flanking or juxtaposed to at least one of

a TCE, for example, between TCE sequences and NS3/4A sequences. Polypeptides encoded by any of the nucleic acids provided herein are also embodiments.

[0016] In some embodiments, nucleic acids encoding an antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence are inserted within a nucleic acid encoding an HCV NS3/4A polypeptide (*e.g.*, **SEQ ID NOs: 1** or **35**) or fragment thereof (*e.g.*, the NS3/4a nucleic acid fragment can comprise, consist of, or consist essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, or 2000 consecutive nucleotides of an NS3 and/or NS4A sequence, such as **SEQ ID NOs: 1** or **35**). Such embodiments can be used as DNA immunogens, which can be delivered to a subject in need of an immune response to the antigen contained therein transdermally (*e.g.*, a transdermal oil or patch), by injection (*e.g.*, hypodermic or ballistic), electroporation (*e.g.*, gene gun, microneedle, needleless, *ex vivo*, MedPulsar™, electroporation with needles or following needleless injection), or any combination thereof. Alternatively, polypeptides encoded by said DNA immunogens can be delivered to a subject in need of an immune response to the antigen. For example, in some embodiments, a nucleic acid encoding an antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence is inserted between the codons of an NS3/4A nucleic acid at sites that do not interrupt protease activity and/or helicase activity such that the expressed fusion peptide retains such catalytic activity and the nucleic acid immunogen is delivered to a subject in need of an immune response to said antigen or TCE by an electroporation system that comprises a needle injection system (*e.g.*, Medpulsar™). Again, aspects disclosed herein include polypeptides that are encoded by said nucleic acids.

[0017] That is, in some embodiments, antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence are inserted between any two contiguous nucleotides between nucleotides 3 and 2059 of an NS3/4A nucleic acid sequence such as **SEQ ID NO: 1**, an NS3/4A variant or consensus nucleic acid sequence (*e.g.*, an NS3/4A sequence developed from a plurality of NS3/4A isotypes, such as **SEQ ID NO: 35**),

or any NS3/4A mutant (for example any nucleic acid encoding an NS3/4A with altered protease activity). Preferably, the nucleic acids encoding the inserted sequences retain the reading frame of the chimeric NS3/4A polypeptide and when the TCE encodes an HCV TCE, the sequence is inserted at a position that is not naturally occurring. In some embodiments, the TCE is not an HCV peptide or peptide fragment. In preferred embodiments, the nucleic acid encoding the TCE can be inserted between nucleotides 1370 and 1548 of **SEQ ID NO:35**, or in an analogous position in another NS3/4A coding sequence. Aspects of the invention also include the polypeptides encoded by the nucleic acid embodiments provided herein.

[0018] Accordingly, the nucleic acids encoding chimeric NS3/4A polypeptides can encode an antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence inserted between any two contiguous amino acids between amino acids 1 and 686 of an NS3/4A polypeptide (*e.g.*, **SEQ ID NO: 2**) an NS3/4A variant polypeptide (*e.g.*, **SEQ ID NO: 36**), or any NS3/4A mutant (for example any NS3/4A with altered protease activity). In preferred embodiments, the antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence inserted between amino acids 453 and 513 of **SEQ ID NO:36** or an analogous position in an NS3/4A polypeptide. The encoded polypeptides of the nucleic acid embodiments described herein are also embodiments.

[0019] In some embodiments, the encoded antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence is juxtaposed to or flanking the HCV NS3/4A polypeptide or fragment thereof. For example, the nucleic acid encoding the antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence can be 5' to the nucleic acid encoding the HCV NS3/4A polypeptide or fragment thereof, such that the TCE is on the N-terminal end of the encoded chimeric polypeptide. Optionally, the nucleic acid encoding the antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and

linker and adjuvant sequence, or TCE and linker and adjuvant sequence can be 3' to the nucleic acid encoding the HCV NS3/4A polypeptide or fragment thereof, such that the TCE is on the C-terminal end of the encoded chimeric polypeptide.

[0020] The antigens and/or TCE used in the platforms described herein can include, but are not limited to, nucleic acids that encode viral antigens, bacterial antigens, parasitic antigens, tumor antigens, and toxins. In some embodiments, the encoded TCE comprises a sequence selected from the group consisting of **SEQ ID NOs: 221-571 and SEQ ID NOs: 809-1011 and SEQ ID NO:1014 and SEQ ID NOs: 1016-1034 and SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328**. In preferred embodiments, the encoded TCE or antigen is obtained from a hepatitis virus, such as an antigen from the Hepatitis A virus (HAV), Hepatitis B virus (HBV), or HCV, HIV, a flu virus, an allergen (such as Birch allergen), or malaria. For example, in some embodiments, the encoded TCE comprises the amino acid sequence of **SEQ ID NO:1014**. Antigens and TCEs that are present on pathogens that infect domestic animals are also embodied. That is, some embodiments include veterinary preparations that comprise a nucleic acid encoding an NS3/4A platform, as described herein, and an antigen present on an animal pathogen (e.g., swine flu, avian flu, or equine flu).

[0021] Some embodiments also concern the chimeric polypeptides encoded by the nucleic acids disclosed herein. In some embodiments, the chimeric polypeptide encoded by the isolated nucleic acid retains NS3A protease activity and/or NS3A helicase activity and/or a protease cleavage site. Optionally, the chimeric polypeptides include a linker or adjuvant polypeptide (e.g., an RNA binding domain, such as poly Arg). For example, in some embodiments, the linker flanks at least one end (*i.e.*, N-terminal or C-terminal end) of the TCE. In some embodiments, a linker comprising one to six alanine and/or glycine residues is flanking or juxtaposed to at least one end of the TCE, such as between NS3/4A amino acid sequences and TCE sequences.

[0022] Also provided herein are NS3/4A chimeric polypeptides that include NS3/4A polypeptides or variants, or fragments thereof and at least one antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence. Optionally, these sequences are inserted within the HCV NS3/4A polypeptide or fragment

thereof. For example, the chimeric NS3/4A polypeptides can consist of, consist essentially of, or comprise an antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence inserted between any two contiguous amino acids between amino acids 1 and 685 of an NS3/4A polypeptide(e.g., **SEQ ID NO: 2**) or NS3/4A variant polypeptide, (e.g., **SEQ ID NO: 36**), or fragment thereof. In preferred embodiments, the chimeric NS3/4A polypeptides can consist of, consist essentially of, or comprise an antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence inserted between amino acids 453 and 513 of **SEQ ID NO:36**, or in an analogous position in any NS3/4A polypeptide.

[0023] In some embodiments, the chimeric NS3/4A polypeptide includes an antigen, TCE, antigen and linker, TCE and linker, antigen and adjuvant sequence, TCE and adjuvant sequence, antigen and linker and adjuvant sequence, or TCE and linker and adjuvant sequence that is juxtaposed to or flanking the NS3/4A polypeptide or a fragment thereof. For example, the TCE can be juxtaposed to or flanking the N-terminal or C-terminal end of the chimeric NS3/4A polypeptide or NS3/4A variant. In preferred embodiments, the chimeric NS3/4A polypeptides retain NS3 protease activity, and/or NS3 helicase activity.

[0024] In some embodiments, the TCE can be derived from antigens such as viral antigens, bacterial antigens, parasitic antigens, tumor antigens, allergens and toxins. For example, in some embodiments, the TCE comprises a sequence selected from the group consisting of **SEQ ID NOs: 221-571** and **SEQ ID NOs:809-1011** and **SEQ ID NO:1014** and **SEQ ID NOs: 1016-1034** and **SEQ ID NOs: 1146-1173** and **SEQ ID NOs: 1210-1328**. In preferred embodiments, the encoded TCE or antigen is obtained from a hepatitis virus, such as an antigen from the Hepatitis A virus (HAV), Hepatitis B virus (HBV), or HCV or HIV, flu, Birch allergens or malaria. For example, in some embodiments, the TCE comprises the amino acid sequence of **SEQ ID NO:1014**. Antigens and TCEs that are present on pathogens that infect domestic animals are also embodied. That is, some embodiments include veterinary preparations that comprise a NS3/4A platform, as described herein, and an antigen present on an animal pathogen (e.g., swine flu, avian flu, or equine flu).

[0025] Another embodiment disclosed herein includes a composition that comprises a recombinant peptide immunogen comprising at least one antigen and a hepatitis C virus (HCV) NS3 protease cleavage site, wherein the HCV NS3 protease cleavage site is joined to the antigen at a position that is not naturally occurring. In some embodiments, the antigen comprises an epitope from a plant, virus, bacteria, or a cancer cell. In other embodiments, the antigen is not a peptide from HCV. In still other embodiments, the antigenic fragment comprises an epitope from birch, peanut, wheat protein, a hepatitis viral protein, a hepatitis B viral protein, or hepatitis B virus core protein (HBcAg). In other embodiments, the antigenic fragment comprises a fragment of an antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328.

[0026] In some aspects, the recombinant peptide immunogen comprises a plurality of NS3 protease cleavage sites. In some embodiments, the NS3 protease cleavage site is chosen from the group consisting of NS3/4A, NS4A/B, NS4B/5A, and NS5A/B. In some embodiments, the HCV NS3 protease cleavage site comprises the sequence: SADLEVVTTSTWVLVGGVL (**SEQ ID NO: 1340**). In other embodiments, the HCV NS3 protease cleavage site comprises the sequence: DEMEECSQHLPYIEQG (**SEQ ID NO: 1341**). In still other embodiments, the HCV NS3 protease cleavage site comprises a sequence from the sequences presented below:

HCV Strain name:	Amino acid sequence:	Junction	SEQ ID:
H-FDA	CMSADLEVVTT↓STWVLVGGVL	NS3/4A	1342
H-AP	CMSADLEVVTT↓STWVLVGGVL	NS3/4A	1343
HCV-1	CMSADLEVVTT↓STWVLVGGVL	NS3/4A	1344
HCV-J	CMSADLEVVTT↓STWVLVGGVL	NS3/4A	1345
HCV-BK	CMSADLEVVTT↓STWVLVGGVL	NS3/4A	1346
HC-J6	CMQADLEVMT↓STWVLVGGVL	NS3/4A	1347
HCV-T	CMSADLEVVTT↓STWVLVGGVL	NS3/4A	1348
HC-J8	CMQADLEIMT↓SSWVLVGGVL	NS3/4A	1349
HCV-JT, JT	CMSAQLEVVT↓STWVLVGGVL	NS3/4A	1350
H-FDA	YQEFDEMEEC↓SQHLPYIEQG	NS4A/4B	1351
H-AP	YQEFDEMEEC↓SQHLPYIEQG	NS4A/4B	1352
HCV-1	YREFDEMEEC↓SQHLPYIEQG	NS4A/4B	1353
HCV-J	YQEFDEMEEC↓ASHLPYIEQG	NS4A/4B	1354
HCV-BK	YQEFDEMEEC↓ASHLPYIEQG	NS4A/4B	1355

HC-J1, 4	YEA F DEMEEC↓ASRAALIEEG	NS4A/4B	1356
HCV-T	YQEFDEMEEC↓ASHLPYIEQG	NS4A/4B	1357
HC-J8	YQAFDEMEEC↓ASKAALIEEG	NS4A/4B	1358
HCV-JT, JT'	YREFDEMEEC↓ASHLPYIEQG	NS4A/4B	1359
H-FDA	WISSECTTPC↓SGSWLRDIWD	NS4B/5A	1360
H-AP	WISSECTTPC↓SGSWLRDIWD	NS4B/5A	1361
HCV-1	WISSECTTPC↓SGSWLRDIWD	NS4B/5A	1362
HCV-J	WINEDCSTPC↓SGSWLKDVWD	NS4B/5A	1363
HCV-BK	WINEDCSTPC↓SGSWLRDVWD	NS4B/5A	1364
HC-J6	WITEDCPIPC↓SGSWLRDVWD	NS4B/5A	1365
HCV-T	WINEDCSTPC↓SGSWLRDVWD	NS4B/5A	1366
HC-J8	WITEDCPVPC↓SGSWLQDIWD	NS4B/5A	1367
HCV-JT	WINEDCSTPC↓SGSWLKDVWD	NS4B/5A	1368
HCV-JT'	WINEDCSTPC↓SGSWLRDVWD	NS4B/5A	1369
H-FDA	GADTEDVVCC↓SMSYTWTGAL	NS5A/5B	1370
H-AP	GADTEDVVCC↓SMSYSWTGAL	NS5A/5B	1371
HCV-1	EANAEDVVCC↓SMSYSWTGAL	NS5A/5B	1372
HCV-J	GEAGEDVVCC↓SMSYTWTGAL	NS5A/5B	1373
HCV-BK	EEASEDVVCC↓SMSYTWTGAL	NS5A/5B	1374
HC-J6	SEEDDSVVCC↓SMSYSWTGAL	NS5A/5B	1375
HCV-T	EEDGEGVICC↓SMSYTWTGAL	NS5A/5B	1376
HC-J8	SDQEDSVICC↓SMSYSWTGAL	NS5A/5B	1377
HCV-JT, JT'	GEASDDIVCC↓SMSYTWTGAL	NS5A/5B	1378
CONSENSUS	D C↓S		
CONSENSUS	E T↓A		

[0027] In other aspects, the recombinant peptide immunogen comprises a plurality of antigenic fragments of a protein assembled in a non-naturally occurring order. In other aspects, the recombinant peptide immunogen comprises a plurality of antigenic fragments of a protein assembled in a naturally occurring order. In still other aspects, the recombinant peptide immunogen comprises a plurality of antigenic fragments from at least two different proteins assembled in a non-naturally occurring order.

[0028] In more aspects, the composition further comprises an NS3/4A peptide. In some embodiments, the NS3/4A peptide comprises a mutation that enhances protease activity. In some embodiments, the mutation is selected from the group consisting of Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly,

Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, and Thr177Ala. In some embodiments, the NS3/4A peptide is joined to the peptide immunogen. In some embodiments, the NS3/4A peptide is C-terminal with respect to the peptide immunogen. In other embodiments, the NS3/4A peptide is N-terminal with respect to the peptide immunogen. In still other embodiments, the NS3/4A peptide is inserted within the peptide immunogen. In other embodiments, the peptide immunogen is inserted within the NS3/4A peptide. In yet other embodiments, the NS3/4A peptide is not joined to the peptide immunogen.

[0029] Another embodiment disclosed herein includes a composition that comprises a nucleic acid encoding a recombinant peptide immunogen comprising an antigen and a hepatitis C virus (HCV) NS3 protease cleavage site, wherein the HCV NS3 protease cleavage site is joined to the antigen at a position that is not naturally occurring. In some embodiments, the antigen comprises an epitope from a plant, virus, bacteria, or cancer cell. In some embodiments, the antigen is not a peptide from HCV. In some embodiments, the antigen is an antigenic fragment of a birch, peanut, wheat protein, an antigenic fragment of a hepatitis viral protein, an antigenic fragment of a hepatitis B virus protein, an antigenic fragment of the hepatitis B virus core protein (HBcAg).

[0030] In some aspects, the recombinant peptide immunogen comprises a plurality of NS3 protease cleavage sites. In some embodiments, the NS3 protease cleavage site is chosen from the group consisting of NS3/4A, NS4A/B, NS4B/5A, and NS5A/B. In some embodiments, the HCV NS3 protease cleavage site comprises the sequence: SADLEVVTSTWVLVGGVL (**SEQ ID NO: 1340**). In other embodiments, the HCV NS3 protease cleavage site comprises the sequence: DMEECSQHLPLYEQG (**SEQ ID NO: 1341**).

[0031] In other aspects, the recombinant peptide immunogen comprises a plurality of antigenic fragments of a protein assembled in a non-naturally occurring order. In other aspects, the recombinant peptide immunogen comprises a plurality of antigenic fragments of a protein assembled in a naturally occurring order. In still other aspects, the recombinant peptide immunogen comprises a plurality of antigenic fragments from at least two different proteins assembled in a non-naturally occurring order. In some embodiments, a

peptide immunogen described herein is not native to hepatitis C. In other embodiments, a peptide immunogen described herein is not native to a hepatitis virus. In other embodiments, a peptide immunogen described herein is not native to influenza.

[0032] In some aspects, the composition further comprises a nucleic acid encoding NS3/4A peptide. In some embodiments, the nucleic acid coding for NS3/4A peptide is codon-optimized for expression in a mammal or bird. In other embodiments, the nucleic acid coding the NS3/4A peptide is codon-optimized for expression in a human, dog, cat, horse, pig, cow, goat, or chicken. In some embodiments, the NS3/4A peptide comprises a mutation that enhances protease activity. In some embodiments, the mutation is selected from the group consisting of Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly, Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, and Thr177Ala. In some embodiments, the nucleic acid encoding NS3/4A peptide is joined to the nucleic acid encoding the peptide immunogen. In yet other embodiments, the nucleic acid encoding NS3/4A peptide is not joined to the peptide immunogen.

[0033] In an alternative aspect, the composition further comprises the NS3/4A peptide. In some embodiments, the NS3/4A peptide comprises a mutation that enhances protease activity. In some embodiments, the mutation is selected from the group consisting of Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly, Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, and Thr177Ala. In some embodiment, the NS3/4A peptide is joined to the peptide immunogen. In some embodiments, the NS3/4A peptide is C-terminal with respect to the peptide immunogen. In other embodiments, the NS3/4A peptide is N-terminal with respect to the peptide immunogen. In still other embodiments, the NS3/4A peptide is inserted within the peptide immunogen. In other embodiments, the peptide immunogen is inserted within the NS3/4A peptide. In yet other embodiments, the NS3/4A peptide is not joined to the peptide immunogen.

[0034] It has been discovered that certain mutations in the NS3 domain of HCV allow for protease cleavage of some substrates but not others. More particularly, it was

found that alanine substitution at positions 1050 and 1060 of the NS3 protease created protease molecules with altered substrate specificity. The HCV NS3 mutants V1050A and Q1060A were found to cleave at the NS3-NS4A junction but were unable to cleave the cellular substrate IPS-1/Cardif/MAVS/VISA. Since cleavage of IPS-1/Cardif/MAVS/VISA by wild-type NS3 reduces the response to the toll-like receptors (TLR-3) and the RIG-I pathway, which in turn impairs interferon (IFN) alpha and beta signaling, it is contemplated that the HCV NS3 V1055A and Q1060A mutants allow for cleavage of the NS3-4A junction without reducing interferon alpha and beta signaling. Type I interferons are central mediators for antiviral responses. Interferon- promoter stimulator 1 (IPS-1) contains an N-terminal CARD-like structure that mediates interaction with the CARD of RIG-I and Mda5, which are cytoplasmic RNA helicases that sense viral infection. 'Knockdown' of IPS-1 by small interfering RNA blocks interferon induction by virus infection. Thus, IPS-1 is an adaptor involved in RIG-I- and Mda5-mediated antiviral immune responses.

[0035] Accordingly, HCV NS3 mutants V1055A and Q1060A, protease active fragments of these molecules containing said mutations, and nucleic acids encoding these molecules are therefore useful in the HCV immunogen fusion proteins or vaccines described herein for induction of an immune response against HCV when elevated interferon alpha and beta signaling (e.g., amounts of IFN-alpha or IFN-beta commensurate with amounts of IFN-alpha or IFN-beta in uninfected cells or an uninfected individual) is desired. Additionally, the HCV NS3 mutants V1050A and Q1060A, protease active fragments of these molecules containing said mutations, and nucleic acids encoding these molecules are useful as platforms for incorporation or attachment of heterologous peptides to which an immune response is desired when elevated interferon alpha and beta signaling (e.g., amounts of IFN-alpha or IFN-beta commensurate with amounts of IFN-alpha or IFN-beta in uninfected cells or an uninfected individual) is preferred. Furthermore, HCV NS3 mutants V1050A and Q1060A, protease active fragments of these molecules containing said mutations, and nucleic acids encoding these molecules can be provided in combination with other immunogenic peptides or nucleic acids encoding immunogenic peptides so as induce an adjuvant activity toward said immunogenic peptides or to otherwise induce an immune response characterized by interferon alpha and beta signaling commensurate with that observed in uninfected cells or an uninfected individual.

[0036] Embodiments comprise, consist, or consist essentially of the peptides (SEQ. ID. NOS.:68, 73, and 1329) or fragments thereof that retain protease activity, nucleic acids encoding these molecules, vectors having said nucleic acids, and cells having said vectors, nucleic acids, or peptides. Additional embodiments include an NS3 or NS3/4A encoding nucleic acid or fragment thereof or corresponding peptide, which comprise a sequence that was optimized for codons most frequently used in humans. That is, more embodiments comprise, consist, or consist essentially of nucleic acids that have been codon optimized for expression in humans, which encode the mutant HCV peptides described herein (e.g., SEQ. ID. NOS.:68, 73, and 1329) or fragments thereof that retain protease activity. Vectors having said codon-optimized nucleic acids, immunogenic preparations, and vaccines having said codon-optimized nucleic acids and vectors and cells having said vectors are also embodiments. Preferred embodiments include DNA immunogens that comprise, consist, or consist essentially of nucleic acids encoding SEQ. ID. NOS 68, 73, and 1329 or a fragment thereof that retain protease activity. These DNA immunogens can be provided to cells by transfection, injection, electroporation, needle electroporation (e.g., Medpulsar or Elgin), gene gun, transdermal application, or intranasal application.

[0037] In one embodiment, specific mutations of the NS3 protease domain can be made and screened to find specific mutants that have little effect, no effect, or heightened effect on protease cleavage at the NS3-NS4a cleavage site while losing the ability to cleave IPS-1 to Δ IPS-1. In a preferred embodiment, amino acids 1053 through 1062 of the NS3/4A gene, corresponding to amino acids 27 through 36 of SEQ ID NOS.:1330-1339 have been identified as affecting the specificity of the NS3 protease domain. Accordingly, mutations in this region of the NS3/4A or fragments containing mutations in this region can be selected for their ability to cleave the NS3-NS4A cleavage site while being unable to cleave IPS-1 to Δ IPS-1.

[0038] A large scale mutational analysis of HCV NS3 domain was undertaken. This project produced a variety of truncated versions of the NS3/4A peptide (e.g., SEQ. ID. NOS.: 12 and 13) and mutants that lacked a proteolytic cleavage site (e.g., SEQ. ID. NOS.: 3-11). Other HCV NS3 mutants were found to have altered protease activity (e.g., SEQ ID NOS: 40-220). For example, some mutants, which have an alanine substitution or a glycine substitution in the NS3 protease domain were found to have an abolished, reduced,

enhanced, or greatly enhanced protease activity. Exemplary mutants include any sequence selected from **SEQ ID NOs: 40-220**. Accordingly, regions of the NS3 domain, which retained protease activity was carefully mapped by mutational analysis. Surprisingly, approximately 87% of the protease residues could be replaced and protease activity was retained.

[0039] Aspects of the present invention include compositions that comprise, consist, or consist essentially of the nucleic acid sequence provided by the sequence of **SEQ. ID. NO.: 35** and/or the peptide sequence provided by the sequence of **SEQ. ID. NO.: 36**. Preferred embodiments, for example, include compositions that comprise, consist or consist essentially of any number of consecutive nucleotides between at least 12- 2112 nucleotides of **SEQ. ID. NO.: 35** or a complement thereof (e.g., 12-15, 15-20, 20-30, 30-50, 50-100, 100-200, 200-500, 500-1000, 1000-1500, 1500-2079, or 1500-2112 consecutive nucleotides). Preferred embodiments also include compositions that comprise, consist or consist essentially of any number of consecutive nucleotides between at least 12- 2112 nucleotides of **SEQ. ID. NO.: 35** or a complement thereof (e.g., at least 3, 4, 6, 8, 10, 12, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 consecutive nucleotides acids of **SEQ. ID. NO.: 35**). Additional embodiments include nucleic acids that comprise, consist, or consist essentially of a sequence that encodes **SEQ. ID. NO.: 36** or a fragment thereof, that is, any number of consecutive amino acids between at least 3-50 amino acids of **SEQ. ID. NO.: 36** (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 consecutive amino acids). Still more embodiments include peptides that comprises, consist, or consist essentially of the sequence of **SEQ. ID. NO.: 36** or a fragment thereof, that is, any number of consecutive amino acids between at least 3-50 amino acids of **SEQ. ID. NO.: 36** (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 consecutive amino acids).

[0040] Other preferred embodiments include compositions that comprise, consist, or consist essentially of any number of consecutive nucleotides between at least 12-2112 nucleotides that encode the polypeptides of any sequence selected from **SEQ ID NOs: 40-220**, wherein the nucleic acid includes the coding sequence for the mutation in the NS3 protease domain of the above NS3/NS4A polypeptides, or a complement thereof, (e.g., 12-15, 15-20, 20-30, 30-50, 50-100, 100-200, 200-500, 500-1000, 1000-1500, 1500-2079, or 1500-2112 consecutive nucleotides).

[0041] Methods of making and using the compositions described herein are also provided. In addition to methods of making the embodied nucleic acids and peptides, other embodiments include methods of making immunogens and/or vaccine compositions that can be used to treat or prevent HCV infection. Some methods are practiced, for example, by mixing an adjuvant with a peptide or nucleic acid antigen (e.g., an HCV peptide or HCV nucleic acid), as described above, so as to formulate a single composition (e.g., a vaccine composition). Preferred methods involve the mixing of ribavirin with an HCV gene or antigen disclosed herein.

[0042] Preferred methods of using the compositions described herein involve providing an animal in need of an immune response to HCV with a sufficient amount of one or more of the nucleic acid or peptide embodiments described herein. By one approach, for example, an animal in need of an immune response to HCV (e.g., an animal at risk or already infected with HCV, such as a human) is identified and said animal is provided an amount of NS3/4A (**SEQ. ID. NO.: 2** or **SEQ. ID. NO.: 36**), a mutant NS3/4A (**SEQ. ID. NOs.: 3-13**), a fragment thereof (e.g., **SEQ. ID. NOs.: 14-26**) or a nucleic acid encoding said molecules that is effective to enhance or facilitate an immune response to the hepatitis viral antigen. Additional methods are practiced by identifying an animal in need of a potent immune response to HCV and providing said animal a composition comprising a peptide comprising an antigen or epitope present on **SEQ. ID. NOs.: 2-27** or **SEQ. ID. NO.: 36** or a nucleic acid encoding said peptides. Particularly preferred methods involve the identification of an animal in need of an immune response to HCV and providing said animal a composition comprising an amount of HCV antigen (e.g., NS3/4A (**SEQ. ID. NO.: 2** or **SEQ. ID. NO.: 36**)), mutant NS3/4A (**SEQ. ID. NOs.: 3-13**), a fragment thereof containing any number of consecutive amino acids between at least 3-50 amino acids (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 consecutive amino acids) of **SEQ. ID. NO.: 2** or **SEQ. ID. NO.: 36** (e.g., **SEQ. ID. NOs.: 14-26**) or a nucleic acid encoding one or more of these molecules that is sufficient to enhance or facilitate an immune response to said antigen. In some embodiments, the composition described above also contains an amount of ribavirin that provides an adjuvant effect.

[0043] Other approaches concern identifying an animal in need of an immune response to HCV and providing an amount of NS3/4A polypeptides with altered protease

activity, or mutations in the NS3 protease domain (e.g., any sequence selected from **SEQ ID NOs: 40-220**, a fragment thereof, or a nucleic acid encoding said molecules that is effective to enhance or facilitate an immune response to the hepatitis viral antigen. Additional methods are practiced by identifying an animal in need of a potent immune response to HCV and providing said animal a composition comprising a peptide comprising an antigen or epitope present on any sequence selected from **SEQ ID NOs: 40-220** or a nucleic acid encoding said peptides. Particularly preferred methods involve the identification of an animal in need of an immune response to HCV and providing said animal a composition comprising an amount of HCV antigen (e.g. any sequence selected from **SEQ ID NOs: 40-220**), a fragment thereof containing any number of consecutive amino acids between at least 3-50 amino acids, wherein the fragment includes the mutation in the NS3 protease domain, (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 consecutive amino acids), or a nucleic acid encoding one or more of these molecules that is sufficient to enhance or facilitate an immune response to said antigen. In some embodiments, the composition described above also contains an amount of ribavirin that provides an adjuvant effect.

[0044] In still more embodiments, for example, a gene gun is used to administer an HCV nucleic acid described herein (e.g., **SEQ. ID. NO.: 35** or fragment thereof, as described above) to a mammalian subject in need of an immune response to HCV. In some embodiments, an amount of ribavirin is mixed with the DNA immunogen prior to delivery with the gene gun. In other embodiments, the DNA immunogen is provided by gene gun shortly before or after administration of ribavirin at or near the same site of DNA inoculation. For example, in some embodiments, a gene gun or a transdermal delivery system is used to administer HCV nucleic acids including nucleic acids encoding the NS3/NS4A polypeptides with altered protease activity. Accordingly, a gene gun or a transdermal delivery system is used to deliver nucleotides encoding the NS3/NS4A polypeptides of any sequence selected from **SEQ ID NOs: 40-220**, or fragments thereof that contain the mutation in the NS3 protease domain, either with or without ribavirin.

[0045] In still other embodiments, transdermal delivery systems are used to deliver an HCV polypeptide described herein to a mammalian subject in need of an immune response to HCV. In some embodiments, an amount of ribavirin is mixed and delivered transdermally with the HCV polypeptide. In other embodiments, an amount of ribavirin is

transdermally delivered to a mammal shortly before or shortly after transdermal delivery of the HCV polypeptide, at or near the same site as the polypeptide. For example, embodiments include transdermal delivery of NS3/NS4A polypeptides having altered protease activity. Thus, in some embodiments, the NS3/NS4A polypeptides of any sequence selected from **SEQ ID NOs: 40-220** are delivered transdermally to a mammalian subject in need of an immune response to HCV, either with or without ribavirin.

[0046] Some embodiments relate to methods of inducing an immune response to Hepatitis C Virus using any of the peptides, nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein.

[0047] Other embodiments relate to methods of inducing an immune response to heterologous antigen joined to or co-administered with the peptides, nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein. In some aspects the heterologous antigen is chemically fused to the peptides, nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein. In other aspects the heterologous antigen is co-administered to the the peptides, nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein. In still other aspects the heterologous antigen is administered before administration of the peptides, nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein. In other aspects the heterologous antigen is administered after administration of the the peptides, nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein.

[0048] In some aspects, nucleic acids encoding heterologous antigens described herein that are joined to or co-administered with the nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein are at least, at least about, less than, or less than about 3 nucleotides, 4 nucleotides, 5 nucleotides, 6 nucleotides, 7 nucleotides, 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22

nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, 30 nucleotides, 31 nucleotides, 32 nucleotides, 33 nucleotides, 34 nucleotides, 35 nucleotides, 36 nucleotides, 37 nucleotides, 38 nucleotides, 39 nucleotides, 40 nucleotides, 41 nucleotides, 42 nucleotides, 43 nucleotides, 44 nucleotides, 45 nucleotides, 46 nucleotides, 47 nucleotides, 48 nucleotides, 49 nucleotides, 50 nucleotides, 55 nucleotides, 60 nucleotides, 65 nucleotides, 70 nucleotides, 75 nucleotides, 80 nucleotides, 85 nucleotides, 90 nucleotides, 95 nucleotides, 100 nucleotides, 110 nucleotides, 120 nucleotides, 130 nucleotides, 140 nucleotides, 150 nucleotides, 160 nucleotides, 170 nucleotides, 180 nucleotides, 190 nucleotides, 200 nucleotides, 250 nucleotides, 300 nucleotides, 350 nucleotides, 400 nucleotides, 450 nucleotides, 500 nucleotides, 550 nucleotides, 600 nucleotides, 650 nucleotides, 700 nucleotides, 750 nucleotides, 800 nucleotides, 850 nucleotides, 900 nucleotides, 950 nucleotides, 1000 nucleotides, 1100 nucleotides, 1200 nucleotides, 1300 nucleotides, 1400 nucleotides, 1500 nucleotides, 1600 nucleotides, 1700 nucleotides, 1800 nucleotides, 1900 nucleotides, 2000 nucleotides, 2500 nucleotides, 3000 nucleotides, 3500 nucleotides, 4000 nucleotides, 4500 nucleotides, 5000 nucleotides, 6000 nucleotides, 7000 nucleotides, 8000 nucleotides, 9000 nucleotides, 10,000 nucleotides in length.

[0049] In some aspects, heterologous antigens described herein that are joined to or co-administered with the nucleic acids, nucleic acids encoding said peptides, peptide fragments, or nucleic acids encoding said peptide fragments described herein are at least, at least about, less than, or less than about 3 amino acids, 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, 8 amino acids, 9 amino acids, 10 amino acids, 11 amino acids, 12 amino acids, 13 amino acids, 14 amino acids, 15 amino acids, 16 amino acids, 17 amino acids, 18 amino acids, 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 23 amino acids, 24 amino acids, 25 amino acids, 26 amino acids, 27 amino acids, 28 amino acids, 29 amino acids, 30 amino acids, 31 amino acids, 32 amino acids, 33 amino acids, 34 amino acids, 35 amino acids, 36 amino acids, 37 amino acids, 38 amino acids, 39 amino acids, 40 amino acids, 41 amino acids, 42 amino acids, 43 amino acids, 44 amino acids, 45 amino acids, 46 amino acids, 47 amino acids, 48 amino acids, 49 amino acids, 50 amino acids, 55 amino acids, 60 amino acids, 65 amino acids, 70 amino acids, 75 amino acids, 80 amino acids, 85 amino acids, 90 amino acids, 95 amino acids, 100 amino acids, 110 amino

acids, 120 amino acids, 130 amino acids, 140 amino acids, 150 amino acids, 160 amino acids, 170 amino acids, 180 amino acids, 190 amino acids, 200 amino acids, 250 amino acids, 300 amino acids, 350 amino acids, 400 amino acids, 450 amino acids, 500 amino acids, 550 amino acids, 600 amino acids, 650 amino acids, 700 amino acids, 750 amino acids, 800 amino acids, 850 amino acids, 900 amino acids, 950 amino acids, 1000 amino acids, 1100 amino acids, 1200 amino acids, 1300 amino acids, 1400 amino acids, 1500 amino acids, 1600 amino acids, 1700 amino acids, 1800 amino acids, 1900 amino acids, 2000 amino acids, 2500 amino acids, 3000 amino acids, 3500 amino acids, 4000 amino acids, 4500 amino acids, 5000 amino acids, 6000 amino acids, 7000 amino acids, 8000 amino acids, 9000 amino acids, 10,000 amino acids in length.

[0050] Still other embodiments relate to methods of inducing an immune response as described herein when cleavage of IPS-1 to Δ IPS-1 is not desired.

[0051] Other embodiments relate to methods of inducing an immune response as described herein wherein repression of IFN α and/or IFN β is not desired. This is particularly useful when raising an immune response to HCV because HCV NS3/4A represses IFN α and IFN β expression through proteolytic cleavage of IPS-1.

[0052] One embodiment relates to a method of enhancing an immune response to a hepatitis C antigen comprising identifying an animal in need of an enhanced immune response to a hepatitis C antigen and providing to said animal a composition comprising a nucleic acid sequence that encodes a peptide comprising an NS3 protease domain that cleaves NS3-NS4A cleavage site but does not cleave IPS-1 to Δ IPS-1.

[0053] Another embodiment relates to a method of enhancing an immune response to a hepatitis C antigen comprising identifying an animal in need of an enhanced immune response to a hepatitis C antigen and providing to said animal a peptide that comprises an NS3 protease domain that cleaves NS3-NS4A cleavage site but does not cleave IPS-1 to Δ IPS-1. In one aspect of the embodiments, the composition further comprises ribavirin. In another aspect of the embodiments, the peptide used is selected from the group consisting of **SEQ ID NO: 68, SEQ ID NO: 73, SEQ ID NO: 1329, SEQ ID NO: 1330, SEQ ID NO: 1331, SEQ ID NO: 1332, SEQ ID NO: 1333, SEQ ID NO: 1334, SEQ ID NO: 1335, SEQ ID NO: 1336, SEQ ID NO: 1337, SEQ ID NO: 1338, and SEQ ID NO: 1339.**

[0054] Another embodiment relates to a method of enhancing an immune response to a hepatitis C antigen comprising identifying an animal in need of an enhanced immune response to a hepatitis C antigen and providing to said animal a composition comprising at least 100 consecutive nucleotides of a nucleic acid sequence that encodes a peptide comprising an NS3 protease domain that cleaves NS3-NS4A cleavage site but does not cleave IPS-1 to Δ IPS-1, wherein said nucleic acid codes for a peptide fragment that retains the ability to cleave the NS3-NS4A cleavage site but not cleave IPS-1 to Δ IPS-1.

[0055] Still another embodiment relates to a method of enhancing an immune response to a hepatitis C antigen comprising identifying an animal in need of an enhanced immune response to a hepatitis C antigen and providing to said animal a composition comprising a peptide comprising at least 34 amino acids of an NS3 protease domain that cleaves NS3-NS4A cleavage site but does not cleave IPS-1 to Δ IPS-1, wherein said peptide fragment retains the ability to cleave the NS3-NS4A cleavage site but not cleave IPS-1 to Δ IPS-1.

[0056] One embodiment relates to a purified or isolated nucleic acid encoding a polypeptide comprising an NS3/NS4A polypeptide that cleaves NS3-NS4A cleavage site but cannot cleave IPS-1 to Δ IPS-1 wherein the polypeptide is SEQ ID NO. 1329.

[0057] In one aspect, the mutant NS3/4A peptide has altered cleavage specificity for host proteins. In another aspect, the nucleic acid is part of a composition. In some aspects, the composition further comprises ribavirin.

[0058] One embodiment relates to a purified or isolated nucleic acid encoding a mutant NS/4A peptide with retained protease activity, wherein said mutant NS3/4A peptide has altered cleavage specificity for host proteins.

[0059] One embodiment relates to a method of enhancing an immune response to a hepatitis C antigen comprising identifying an animal in need of an enhanced immune response to a hepatitis C antigen; and administering to said animal a composition a purified or isolated nucleic acid encoding a mutant NS/4A peptide with retained protease activity, wherein said mutant NS3/4A peptide has altered cleavage specificity for host proteins. In one aspect, the composition further comprises ribavirin. In another aspect of the method, ribavirin is co-administered. In yet another aspect, the co-administration of ribavirin is immediately before or immediately after the administration of said composition. In yet

another aspect of the embodiments described herein, the site of said administration, or a region proximal to said site of administration, is contacted with an electrode configured for electroporation of a nucleic acid.

[0060] Vectors that include the isolated nucleic acids or that encode the isolated polypeptides described herein are also embodiments. Compositions that include the nucleic acids, polypeptides, and vectors described herein (e.g., vials, tubes, hypodermic needles, electroporation devices, oils, and transdermal delivery compositions) are also embodiments. Cells that include these vectors are also provided herein. For example, in some embodiments, the nucleic acids described herein are inserted into an expression vector such as a pVAX™ expression vector or are inserted into a Semliki forest virus vector and these expression constructs are provided to a subject in need of an immune response to the antigen.

[0061] Stated differently, several embodiments described herein concern nucleic acids that encode a recombinant HCV NS3/4A chimeric molecule, wherein the nucleic acid encodes four domains (domain W, domain X, domain Y, and domain Z). Domain W can encode an NS3 protease domain prepared as described herein (e.g., a mutant or modified version), or fragment thereof, domain X can encode an NS3 helicase domain, or a fragment thereof, and domain Y can encode an NS4A co-factor domain, or a fragment thereof. Domain Z can encode an antigen, an epitope of a pathogen, a TCE, and/or the aforementioned molecules with or without a linker and/or adjuvant sequence. In preferred embodiments, the recombinant HCV NS3/4A chimeric polypeptide retains catalytic activity (e.g., protease, helicase, or protease and helicase activity). In preferred embodiments, domain Z is in a position within the recombinant nucleic acid and encoded chimeric polypeptide, which is non-naturally-occurring (e.g., when Z encodes an HCV antigen).

[0062] In some embodiments, the W domain comprises the nucleic acid sequence of residues **1-551** of an NS3/4A sequence such as **SEQ ID NOs: 1** or **35**, or an analogous sequence of any NS3/4A nucleic acid, or a fragment thereof (e.g., said fragment can comprise, consist of, or consist essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, or 300 consecutive nucleotides of the protease domain of **SEQ. ID. Nos. 1** or **35**). In some embodiments, the X domain can comprise the nucleic acid sequence of residues **218-1568** of **SEQ ID NOs: 1** or **35**, or an analogous sequence of any NS3/4A nucleic acid, or a fragment thereof (e.g., said

fragment can comprise, consist of, or consist essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, or 300 consecutive nucleotides of the helicase domain of **SEQ. ID. Nos. 1 or 35**). In some embodiments, the Y domain can comprise the nucleic acid sequence of residues **1569-2069** of **SEQ ID NOs: 1 or 35**, or an analogous sequence of any NS3/4A nucleic acid, or a fragment thereof (e.g., said fragment can comprise, consist of, or consist essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, or 350 consecutive nucleotides of the NS4A domain of **SEQ. ID. Nos. 1 or 35**). In preferred embodiments, the Z domain can comprise the nucleic acid sequence of an antigen, preferably a TCE, and more preferably a sequence selected from the group consisting of **SEQ ID NOs: 221-571** and **SEQ ID NOs:809-1011** and **SEQ ID NO:1014**.

[0063] In some embodiments, the Z domain is located within or flanking (e.g., juxtaposed or immediately adjacent to) said W domain. For example, the nucleic acid encoding the Z domain can place the encoded antigen between or next to any two contiguous amino acids between amino acids 1 181 of said W domain of said chimeric polypeptide.

[0064] In some embodiments, the Z domain is located within or flanking (e.g., juxtaposed or immediately adjacent to) the X domain. For example, the Z domain can place the encoded antigen between or next to any two contiguous amino acids between amino acids 1 and 450 of the X domain of the chimeric polypeptide. Preferably, the Z domain is located between amino acids 383 and 450 of said X domain.

[0065] Optionally, the Z domain is located within or flanking (e.g., juxtaposed or immediately adjacent to) said Y domain. For example, the Z domain can place the encoded antigen between or next to amino acids 1 and 2, 2 and 3, 3 and 4, 4 and 5, 5 and 6, 6 and 7, 7 and 8, 8 and 9, 9 and 10, 10 and 11, 11 and 12, 12 and 13, 13 and 14, 14 and 15, 15 and 16, 16 and 17, 17 and 18, 18 and 19, 19 and 20, 20 and 21, 21 and 22, 22 and 23, 23 and 24, 24 and 25, 25 and 26, 26 and 27, 27 and 28, 28 and 29, 29 and 30, 30 and 31, 31 and 32, 32 and 33, 33 and 34, 34 and 35, 35 and 36, 36 and 37, 37 and 38, 38 and 39, 39 and 40, 40 and 41, 41 and 42, 42 and 43, 43 and 44, 44 and 45, 45 and 46, 46 and 47, 47 and 48, 48 and 49, 49 and 50, 50 and 51, 51 and 52, 52 and 53, or 53 and 54 of the Y domain of the chimeric polypeptide.

[0066] That is, aspects of the invention concern a composition that comprises, consists of, or consists essentially of an isolated nucleic acid provided by any one of the formulas WXYZ, WXZY, WYZX, WYXZ, WZXY, WZYX, XWYZ, XWZY, XYZW, XYWZ, XZWY, ZXYW, YXWZ, YXZW, YWZX, YWXZ, YZXW, YZWX, ZXYW, ZXWY, ZYWX, ZYXW, ZWXY, ZWYX wherein:

[0067] W encodes a protease domain (e.g., an HCV NS3 protease domain prepared as described herein, such as a mutant with enhanced or altered protease activity or substrate specificity) or a fragment thereof, wherein said fragment comprises, consists of, or consists essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, or 300 consecutive nucleotides of the protease domain of **SEQ. ID. Nos. 1 or 35** (e.g., residues **1-551** of **SEQ ID NO:35**, or an analogous position in any NS3/4A nucleic acid);

[0068] X encodes a helicase domain (e.g., an HCV helicase domain) or a fragment thereof, wherein said fragment comprises, consists of, or consists essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, or 300 consecutive nucleotides of the helicase domain of **SEQ. ID. Nos. 1 or 35** (e.g., residues 218-1568 of **SEQ ID NO:35**, or an analogous position in any NS3/4A nucleic acid);

[0069] Y encodes an enhancer domain (e.g., an HCV NS4A domain) or a fragment thereof, wherein said fragment comprises, consists of, or consists essentially of about at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, or 300 consecutive nucleotides of the NS4A domain of **SEQ. ID. Nos. 1 or 35** (e.g., residues 1569-2069 of **SEQ ID NO:35**, or an analogous position in any NS3/4A nucleic acid); and

[0070] Z encodes an antigen (e.g., an antigen of a virus, bacteria, toxin, or cancer cell, such as a TCE provided by a sequence selected from the group consisting of **SEQ ID NOs: 221-571, SEQ ID NOs:809-1011, SEQ ID NO:1014, SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173, and SEQ ID NOs: 1210-1328**), with the proviso that Z is not in a position that is naturally occurring in HCV (e.g., when Z is an HCV antigen, the antigen is inserted at a position that is not naturally occurring in HCV).

[0071] Methods of making and using the compositions described herein are also provided. In addition to methods of making the embodied nucleic acids and polypeptides, other embodiments include method of making immunogens and/or vaccine compositions. Preferably immunogenic compositions or vaccine compositions comprise a pharmaceutically acceptable carrier in addition to the nucleic acids and/or polypeptides described herein. Some methods are practiced, for example, by mixing an adjuvant with a nucleic acid or polypeptide as described above, so as to formulate a single composition (e.g., an immunogenic composition or a vaccine). Preferred methods involve the mixing of ribavirin with the nucleic acids or encoded polypeptides described herein. (See e.g., US Patent No. 6,680,059, and US Patent No. 6,858,590, hereby expressly incorporated by reference in their entirety.)

[0072] Preferred methods of using the compositions described herein involve providing an animal (e.g., a mammal such as a human or a domestic animal) in need of an immune response to an antigen with a sufficient amount of one or more of the nucleic acid and/or polypeptide compositions described herein, wherein the TCE or antigen present in the composition is derived from said antigen/target (e.g., an antigen present on a pathogen, such as a virus like hepatitis, HIV, or the flu). In some embodiments, the animal, preferably a mammal, collectively referred to as a subject, is identified as a subject in need of an immune response to an antigen or a pathogen comprising an antigen, and said identified subject is provided a therapeutically effective amount of one or more of the NS3/4A platforms comprising said antigen. Optionally, the immune response of said identified subject to said platform comprising said antigen is measured.

[0073] In still more embodiments, a gene gun or electroporation device (e.g., Medpulsar™) is used to introduce the nucleic acids described herein to a mammalian subject in need of an immune response to an agent, wherein the nucleic acids encode a TCE specific to the agent. In some embodiments, an amount of an adjuvant, such as ribavirin, is mixed with the nucleic acid immunogen prior to delivery. In other embodiments, the nucleic acid immunogen is provided shortly before or after administration of ribavirin or at the same site of nucleic acid inoculation. Other embodiments relate to an isolated nucleic acid comprising a nucleic acid sequence encoding a TCE, wherein said nucleic acid sequence encoding said TCE is inserted within a nucleic acid sequence comprising, consisting essentially of, or

consisting of one of the following **SEQ ID NOs: 1, 35, or 573-806**, and wherein said isolated nucleic acid operably encodes a polypeptide that retains catalytic activity. These embodiments can be delivered using electroporation methods, as described above, with or without an adjuvant, such as ribavirin.

[0074] SEQ ID NOs: 1174-1198 correspond to nucleic acid and peptide constructs of fragments of the HBcAg with or without NS3 protease cleavage sites joined to the NS3/4A platform. SEQ ID NOs: 1174-1198 initially correspond to all parts of possible constructs presented therein, including the amino acid sequence for HBcAg, the NS3/4A protease cleavage site, the NS4A/B protease cleavage site, and the NS3/4A platform.

[0075] SEQ ID NOs: 1181-1182 correspond to a functional NS3 protease joined to NS4A via a mutant NS3/4A protease cleavage site. The NS3/4A platform is joined to the HBcAg without any NS3 protease cleavage sites. SEQ ID NOs: 1181-1182, corresponding to FIGURE 1A, will have an active protease (NS3) that is unable to cleave itself.

[0076] SEQ ID NOs: 1183-1184 correspond to a mutant non-functional NS3 protease joined to NS4A via an NS3/4A protease cleavage site. The NS3/4A platform is joined to the HBcAg without any NS3 protease cleavage sites. SEQ ID NOs: 1183-1184, corresponding to FIGURE 1B, will have an inactive protease (NS3) that is unable to cleave itself through the functional protease cleavage site.

[0077] SEQ ID NOs: 1185-1186 correspond to a functional NS3 protease joined to NS4A via an NS3/4A protease cleavage site. The NS3/4A platform is joined to the HBcAg without any NS3 protease cleavage sites. SEQ ID NOs: 1185-1186, corresponding to FIGURE 1C, will have an active protease (NS3) that is able to cleave itself but will not cleave any other portion of the peptide as there are no other protease cleavage sites available. Accordingly, the products created by NS3 cleavage include the NS3 protein and the NS4A joined to the HBcAg.

[0078] SEQ ID NOs: 1187-1188 correspond to a functional NS3 protease joined to NS4A via an NS3/4A protease cleavage site. The NS3/4A platform is joined to the HBcAg via an NS4A/B protease cleavage site. SEQ ID NOs: 1187-1188, corresponding to FIGURE 1D, will have an active protease (NS3) that is able to cleave itself and cleave the NS4A from the HBcAg via the NS4A/B protease cleavage site. Accordingly, the products created by NS3 cleavage include the NS3 protein, the NS4A protein, and the HBcAg peptide.

[0079] SEQ ID NOs: 1189-1190 correspond to a functional NS3 protease joined to NS4A via an NS3/4A protease cleavage site. The NS3/4A platform is joined to the HBcAg via an NS4A/B protease cleavage site. The HBcAg contains NS3/4A protease cleavage sites within the peptide, particularly between amino acids 44 and 45, between amino acids 87 and 88, as well as between amino acids 141 and 142. SEQ ID NOs: 1189-1190, corresponding to FIGURE 1E, will have an active protease (NS3) that is able to cleave itself and cleave the NS4A from the HBcAg via the NS4A/B protease cleavage site. Additionally, the NS3 protease will cleave the HBcAg between amino acids 44 and 45, between amino acids 87 and 88, as well as between amino acids 141 and 142 via the NS3/4A protease cleavage site. Accordingly, the products created by NS3 cleavage include the NS3 protein, the NS4A protein, and the fragments of the HBcAg peptide corresponding to amino acids 1-44, amino acids 45-87, amino acids 88-141, and amino acids 142-183.

[0080] SEQ ID NOs: 1191-1198 correspond to a functional NS3 protease joined to NS4A via an NS3/4A protease cleavage site. The NS3/4A platform is joined to the HBcAg via an NS4A/B protease cleavage site. The HBcAg is shuffled with NS3/4A protease cleavage sites separating the shuffled fragments. SEQ ID NOs: 1191-1198, corresponding to FIGURES 1F-1I, will have an active protease (NS3) that is able to cleave itself and cleave the NS4A from the HBcAg via the NS4A/B protease cleavage site. Additionally, the NS3 protease will cleave the HBcAg shuffled fragments at the site of the NS3/4A protease cleavage site. Accordingly, the products created by NS3 cleavage include the NS3 protein, the NS4A protein, and the fragments of the HBcAg peptide.

[0081] SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 correspond to antigenic peptide sequences from allergens and infectious diseases. The peptides represented can be used as part of an immunogenic composition designed to raise an immune response against the antigen from which they originate. In another embodiment, the nucleic acids coding for the antigenic peptide sequence can be used as part of DNA vaccine designed to raise an immune response against the antigen from which they originate after administration.

[0082] SEQ ID NOs: 1122-1145 correspond to antigenic peptide sequences containing the NS3/4A protease cleavage site inserted at various sites within the peptide sequence. Although the SEQ ID NOs: 1122-1145 recite only the NS3/4A protease cleavage

site, any NS3 protease cleavage site (e.g., NS4A/B protease cleavage site) can be used. The protease cleavage sites are cleaved by an NS3 peptide, which can be administered in peptide or nucleic acid form pre-administration, post-administration, or peri-administration of the antigenic sequence containing the NS3 protease cleavage site(s). The protease cleavage sites improve antigen processing within antigen presenting cells and facilitate a heightened cell-mediated immune response. Although the antigenic sequences of SEQ ID NOs: 1122-1145 contain naturally-ordered antigen fragments separated by an NS3 protease cleavage site, the antigenic fragments can be ordered randomly as in the shuffled fragments of HBcAg separated by NS3 protease cleavage sites seen in SEQ ID NOs: 1174-1198.

[0083] SEQ ID NOs: 1098-1121 correspond to the codon-optimized (human) nucleic acid sequence encoding antigenic peptide sequences containing the NS3/4A protease cleavage site inserted at various sites within the peptide sequence. Although SEQ ID NOs: 1098-1121 encode only the NS3/4A protease cleavage site, any NS3 protease cleavage site (e.g., NS4A/B, NS4B/5A, and NS5A/5B protease cleavage sites) can be used. The protease cleavage sites are cleaved by an NS3 peptide, which can be administered as a peptide or nucleic acid pre-administration, post-administration, or peri-administration of the codon optimized nucleic acid coding the antigenic sequence containing the NS3 protease cleavage site(s). The protease cleavage sites improve antigen processing within antigen presenting cells and facilitate a heightened cell-mediated immune response. Although the codon optimized nucleic acids corresponding to SEQ ID NOs: 1098-1121 encode antigenic sequences containing naturally-ordered antigen fragments separated by an NS3 protease cleavage site, the antigenic fragments can be ordered randomly as in the shuffled fragments of HBcAg separated by NS3 protease cleavage sites seen in SEQ ID NOs: 1174-1198.

[0084] SEQ ID NOs: 1059-1097 correspond to the NS3/4A peptide linked to antigenic peptide sequences containing NS3/4A protease cleavage site inserted at various sites within the peptide sequence. Although SEQ ID NOs: 1059-1097 encode only the NS3/4A protease cleavage site, any NS3 protease cleavage site (e.g., NS4A/B, NS4B/5A, and NS5A/5B protease cleavage sites) can be used. The protease cleavage sites improve antigen processing within antigen presenting cells and facilitate a heightened cell-mediated immune response. Although the antigenic sequences of SEQ ID NOs: 1059-1097 contain naturally-ordered antigen fragments separated by an NS3 protease cleavage site, the antigenic

fragments can be ordered randomly as in the shuffled fragments of HBcAg separated by NS3 protease cleavage sites seen in SEQ ID NOs: 1174-1198.

[0085] SEQ ID NOs: 1035-1058 correspond to the codon-optimized (human) nucleic acid sequence encoding NS3/4A peptide linked to antigenic peptide sequences containing the NS3/4A protease cleavage site inserted at various sites within the peptide sequence. Although SEQ ID NOs: 1035-1058 encode only the NS3/4A protease cleavage site, any NS3 protease cleavage site (e.g., NS4A/B, NS4B/5A, and NS5A/5B protease cleavage sites) can be used. The protease cleavage sites improve antigen processing within antigen presenting cells and facilitate a heightened cell-mediated immune response. Although the codon optimized nucleic acids presented in SEQ ID NOs: 1035-1058 encode antigenic sequences containing naturally-ordered antigen fragments separated by an NS3 protease cleavage site, the antigenic fragments can be ordered randomly as in the shuffled fragments of HBcAg separated by NS3 protease cleavage sites seen in SEQ ID NOs: 1174-1198.

[0086] SEQ ID NO: 1175 corresponds to the wild type sequence of HBcAg and SEQ ID NOs: 1176 and 1393 corresponds to the codon optimized sequence of HBcAg. SEQ ID NOs: 1199-1209 correspond to mutant NS3/4A peptides having altered substrate specificity.

[0087] In some embodiments, antigenic fragments of HBcAg are administered to a subject to raise an immune response. In other embodiments, a nucleic acid encoding an antigenic fragment of HBcAg is administered to a subject to raise an immune response. In a preferred aspect of the embodiment, the nucleic acid is codon optimized for expression in a human. In some embodiments, antigenic fragments of HBcAg are separated by an NS3/4A protease cleavage site.

[0088] In some embodiments a nucleic acid encoding a fragment of **SEQ ID NO. 1393**, arranged in a non-naturally occurring order, is administered to a subject to raise an immune response. In a preferred embodiment, the fragments constitute the entire sequence of **SEQ ID NO. 1393**. In some embodiments, the fragments of SEQ ID NO. 1393 are separated by an NS3/4A protease cleavage site.

[0089] In some embodiments the antigenic fragments of HBcAg are between 5 to 100 amino acids in length, in a preferred embodiment, the antigenic fragments of HBcAg are

between 20 to 70 amino acids in length, in a more preferred embodiment, the antigenic fragments are between 30 to 50 amino acids in length.

BRIEF DESCRIPTION OF THE DRAWINGS

[0090] FIGURES 1A-1I depict various constructs containing the NS3/4A platform and the HBcAg containing NS3 protease cleavage sites.

[0091] FIGURE 2 is a graph showing the antibody titer in H-2^d mice against NS3 as a function of time after the first intra muscular immunization. Diamonds denote antibody titer in mice immunized with NS3/4A-pVAX and squares denote antibody titer in mice immunized with NS3-pVAX.

[0092] FIGURE 3A and 3B show the mean NS3-specific antibody responses primed by gene gun immunisations with 4µg wtNS3/4A-pVAX1 and coNS3/4A-pVAX1, or *s.c.* injection of 10⁷ wtNS3/4A-SFV particles in groups of ten H-2^d mice (FIGURE 3A). All mice were immunized at weeks zero and four. Values are given as mean end-point antibody titres (± SD.). Also shown FIGURE 3B are the IgG subclass patterns from groups of five mice immunized twice with wtNS3/4A-pVAX1 given *i.m.*, coNS3/4A-pVAX1 given *i.m.* or by gene gun (gg), and wtNS3/4A-SFV given *s.c.* Values are given as mean end-point antibody titres (± SD.). A “***” sign indicates a statistical difference of $p < 0.01$, a “**” sign indicates a difference of $p < 0.05$, and NS (not significant) indicates no statistical difference (Mann-Whitney). Also given is the titer ratio obtained by dividing the mean endpoint titre of IgG2a antibodies to NS3 by the mean endpoint titre IgG1 antibodies to NS3. A high ratio (>3) indicates a Th1-like response and a low ratio (<0.3) indicates a Th2-like response, whereas values within a three-fold difference from 1 (0.3 to 3) indicates a mixed Th1/Th2 response.

[0093] FIGURE 4A shows the *in vivo* protection conferred by one gene gun immunization of NS3/4A-pVAX1 (4µg) or MSLF1-pVAX1 (4µg). Mice were immunized with the respective plasmid and 14 days later the mice were challenged with an NS3/4A expressing SP2/0 cell line (approximately 10⁶ cells/mouse). Tumor size was then measured through the skin daily following day 6 post-challenge and the data plotted. FIGURE 4B shows the *in vivo* protection conferred by two gene gun immunizations of NS3/4A-pVAX1 (4µg) or MSLF1-pVAX1 (4µg). Mice were immunized with the respective plasmid at weeks

zero and week four and, 14 days after the last immunization, the mice were challenged with an NS3/4A expressing SP2/0 cell line (approximately 10^6 cells/mouse). Tumor size was then measured through the skin daily following day 6 post-challenge and the data plotted.

[0094] FIGURE 5 shows the *in vivo* protection conferred by three gene gun immunizations of NS3/4A-pVAX1 (4 μ g) or MSLF1-pVAX1 (4 μ g). Mice were immunized with the respective plasmid at weeks zero, week four, and week eight and, 14 days after the last immunization, the mice were challenged with an NS3/4A expressing SP2/0 cell line (approximately 10^6 cells/mouse). Tumor size was then measured through the skin daily following day 6 post-challenge and the data plotted.

[0095] FIGURE 6A is a graph showing the percentage of specific CTL-mediated lysis of SP2/0 target cells as a function of the effector to target ratio. Phosphate Buffered Saline (PBS) was used as a control immunogen.

[0096] FIGURE 6B is a graph showing the percentage specific CTL-mediated lysis of SP2/0 target cells as a function of the effector to target ratio. Plasmid NS3/4A-pVAX was used as the immunogen.

[0097] FIGURE 7A is a graph showing the response of naive splenic T cells that were stimulated with peptide coated RMA-S cells. The naive splenic T cells were obtained from C57/BL6 mice.

[0098] FIGURE 7B is a graph showing the response of splenic T cells that were restimulated with peptide coated RMA-S cells. The splenic T cells were obtained from C57/BL6 mice that were provided a single 4 μ g dose of MSLF1-pVAX1.

[0099] FIGURE 7C is a graph showing the response of splenic T cells that were restimulated with peptide coated RMA-S cells. The splenic T cells were obtained from C57/BL6 mice that were provided a single 4 μ g dose of NS3/4A-pVAX1.

[0100] FIGURE 7D is a graph showing the response of naive splenic T cells that were stimulated with peptide coated RMA-S cells. The naive splenic T cells were obtained from C57/BL6 mice.

[0101] FIGURE 7E is a graph showing the response of splenic T cells that were restimulated with peptide coated RMA-S cells. The splenic T cells were obtained from C57/BL6 mice that were provided two 4 μ g doses of MSLF1-pVAX1.

[0102] FIGURE 7F is a graph showing the response of splenic T cells that were restimulated with peptide coated RMA-S cells. The splenic T cells were obtained from C57/BL6 mice that were provided two 4µg doses of NS3/4A-pVAX1.

[0103] FIGURE 7G is a graph showing the response of naive splenic T cells that were stimulated with NS3/4A expressing EL-4 cells. The naive splenic T cells were obtained from C57/BL6 mice.

[0104] FIGURE 7H is a graph showing the response of splenic T cells that were restimulated with NS3/4A expressing EL-4 cells. The splenic T cells were obtained from C57/BL6 mice that were provided a single 4µg dose of MSLF1-pVAX1.

[0105] FIGURE 7I is a graph showing the response of splenic T cells that were restimulated with NS3/4A expressing EL-4 cells. The splenic T cells were obtained from C57/BL6 mice that were provided a single 4µg dose of NS3/4A-pVAX1.

[0106] FIGURE 7J is a graph showing the response of naive splenic T cells that were stimulated with NS3/4A expressing EL-4 cells. The naive splenic T cells were obtained from C57/BL6 mice.

[0107] FIGURE 7K is a graph showing the response of splenic T cells that were restimulated with NS3/4A expressing EL-4 cells. The splenic T cells were obtained from C57/BL6 mice that were provided two 4µg doses of MSLF1-pVAX1.

[0108] FIGURE 7L is a graph showing the response of splenic T cells that were restimulated with NS3/4A expressing EL-4 cells. The splenic T cells were obtained from C57/BL6 mice that were provided two 4µg doses of NS3/4A-pVAX1.

[0109] FIGURE 8A shows a flow cytometric quantification of the precursor frequency of NS3/4A-specific CD8⁺ T cells using peptide-loaded H-2D^b:Ig fusion protein. In a) the mean % NS3-specific CD8⁺ T cells from groups of five mice immunized twice with wtNS3-pVAX1, wtNS3/4A-pVAX1, or coNS3/4A-pVAX1 using gene gun is shown. A “*” sign indicates a difference of $p < 0.05$, and NS (not significant) indicates no statistical difference (Mann-Whitney). FIGURE 8B, also shown, represents the raw data from representative individual mice from the groups listed above (e, f, and h), as well as from individual mice immunized once with coNS3/4A-pVAX1 (b) or wtNS3/4A-SFV (c). In (d) and (g), non-immunized control mice from the different experiments have been given. In (i) and (j) the splenocytes were restimulated for five days with the NS3-peptides prior to

analysis. A total of 150,000-200,000 data points were collected and the percentage of CD8+ cells stained for H-2D^b:Ig are indicated in the parentheses in each dot-plot.

[0110] FIGURES 9A and 9B show the priming of *in vitro* detectable CTLs in H-2^b mice by gene gun immunization of the wtNS3-pVAX1, wtNS3/4A, and coNS3/4A plasmids, or *s.c.* injection of wtNS3/4A-SFV particles. Groups of five to 10 H-2^b mice were immunized once (FIGURE 9A) or twice (FIGURE 9B). The percent specific lysis corresponds to the percent lysis obtained with either NS3-peptide coated RMA-S cells (upper panel in (FIGURE 9A) and (FIGURE 9B) or NS3/4A-expressing EL-4 cells (lower panel in a and b) minus the percent lysis obtained with unloaded or non-transfected EL-4 cells. Values have been given for effector to target (E:T) cell ratios of 60:1, 20:1 and 7:1. Each line indicates an individual mouse.

[0111] FIGURE 10A shows the specificity of tumor inhibiting immune responses primed by gene gun immunization. Groups of ten C57BL/6 mice were either left untreated or were given two monthly immunizations with 4µg of coNS3/4A-pVAX1. Two weeks after last immunization, mice were injected sub cutaneously with the parental EL-4 cell line or 10⁶ NS3/4A-expressing EL-4 cells. Tumor sizes were measured through the skin at days 6, 7, 10, 11, 12, and 14 after tumour injection. In FIGURE 10B the *in vivo* functional effector cell population was determined in groups of 10 C57BL/6 mice immunized twice with the coNS3/4A-pVAX1 plasmid using gene gun. In two groups either CD4+ or CD8+ T cells were depleted by administration of monoclonal antibodies one week prior to, and during, challenge with the NS3/4A-expressing EL-4 cell line. Tumor sizes were measured through the skin at days 5, 6, 8, 11, 13, 14, and 15 after tumour injection. Values have been given as the mean tumor size ± standard error. A “***” sign indicates a statistical difference of $p < 0.01$, a “*” sign indicates a difference of $p < 0.05$, and NS (not significant) indicates no statistical difference (area under the curve values compared by ANOVA).

[0112] FIGURE 11 shows an evaluation of the ability of different immunogens to prime HCV NS3/4A-specific tumor-inhibiting responses after a single immunization. Groups of ten C57BL/6 mice were either left untreated or were given one immunization with the indicated immunogen (4 µg DNA using gene gun in (a), (b), (c), (g), and (h); 10⁷ SFV particles *s.c.* in d; 100 µg peptide in CFA *s.c.* in (e); and 20µg rNS3 in CFA *s.c.* in (f). Two weeks after last immunization, mice were injected sub cutaneously with 10⁶ NS3/4A-

expressing EL-4 cells. Tumor sizes were measured through the skin at days 6 to 19 after tumor injection. Values have been given as the mean tumor size $\pm$ standard error. In (a) to (e), as a negative control the mean data from the group immunized with the empty pVAX plasmid by gene gun has been plotted in each graph. In (f) to (h) the negative controls were non-immunized mice. Also given is the p value obtained from the statistical comparison of the control with each curve using the area under the curve and ANOVA.

[0113] FIGURES 12A, 12B, and 12C show the comparative efficiency of gene gun delivered wtNS3/4A-pVAX1 and coNS3/4A-pVAX1 plasmids in priming tumor inhibiting immune responses. Groups of ten BALB/c mice were either left untreated or were given one (FIGURE 12A), two (FIGURE 12B) or three (FIGURE 12C) monthly immunisations with 4 μ g of plasmid. Two weeks after last immunization, mice were injected sub cutaneously with 10⁶ NS3/4A-expressing SP2/0 cells. Tumor sizes were measured through the skin at days 6, 8, 10, 11, 12, 13, and 14 after tumor injection. Values have been given as the mean tumor size $\pm$ standard error. A “***” sign indicates a statistical difference of $p < 0.01$, a “*” sign indicates a difference of $p < 0.05$, and NS (not significant) indicates no statistical difference (area under the curve values compared by ANOVA).

[0114] FIGURE 13 shows the effect of therapeutic vaccination with the coNS3/4A plasmid using the gene gun. Groups of ten C57BL/6 mice were inoculated with 10⁶ NS3/4A-EL4 cells. One group had been immunized once with 4 μ g coNS3/4A DNA using a gene gun two weeks prior to challenge (positive control), one group was immunized the same way six days after tumor inoculation, and one group was immunized 12 days after tumor inoculation. One group was not immunized (negative control). Tumor sizes were measured through the skin at days 6, 10, 11, 12, 13, 14, 18, 19, and 20 after tumour injection. Values have been given as the mean tumor size $\pm$ standard error. A “***” sign indicates a statistical difference of $p < 0.01$, a “*” sign indicates a difference of $p < 0.05$, and NS (not significant) indicates no statistical difference (area under the curve values compared by ANOVA).

[0115] FIGURE 14 is a graph showing the humoral response to 10 and 100 μ g recombinant Hepatitis C virus (HCV) non structural 3 protein (NS3), as determined by mean end point titres, when a single dose of 1mg of ribavirin was co-administered.

[0116] FIGURE 15 is a graph showing the humoral response to 20 μ g recombinant Hepatitis C virus (HCV) non structural 3 protein (NS3), as determined by mean end point titres, when a single dose of 0.1, 1.0, or 10mg of ribavirin was co-administered.

[0117] FIGURE 16 is a graph showing the effects of a single dose of 1mg ribavirin on NS3-specific lymph node proliferative responses, as determined by *in vitro* recall responses.

[0118] FIGURE 17 shows the location of amino acid residues in the NS3A protease that affect protease cleavage. Versions of NS3/NS4A-pVAX were constructed to encode proteins in which each amino acid of the shown sequence other than the alanine residues was substituted with an alanine residue. Each alanine residue was substituted with a glycine residue. The encoded proteins were analyzed for protease activity. The red color indicates the 16 mutations which resulted in a protein that lacked all protease activity. The dark blue color indicates the 3 mutations which resulted in a protein that exhibited greatly enhanced protease activity compared to wtNS3/NS4A.

[0119] FIGURE 18 depicts the protease activity of the NS3 protease domain in which alanine or glycine was substituted for each protease-domain residue. Each mutant was tested for protease activity after translation. The upper band corresponds to the noncleaved NS3/4A fusion protein and the lower band corresponds to the free NS3 protein. A single or clearly dominant peak indicates destroyed or enhanced protease activity, compared with the dual peak appearance of the wt NS3/4A gene.

[0120] FIGURES 19A and B depict SDS Page gels where IPS-1 cleavage by particular mutants are visualized.

[0121] FIGURE 20A is a graph showing the response of splenic T cells that were restimulated with peptide coated RMA-S cells. The splenic T cells were obtained from C57/BL6 mice that were provided a single 100 μ g dose of HBcAg-pVAX1 intramuscularly at week 0 and week 4, as indicated.

[0122] FIGURE 20B is a graph showing the response of splenic T cells that were restimulated with peptide coated RMA-S cells. The splenic T cells were obtained from C57/BL6 mice that were provided a single 4 μ g dose of HBcAg-pVAX1 with a gene gun at week 0 and week 4, as indicated.

[0123] FIGURE 21 shows the location of amino acid residues in the HCV NS3 protease that affect protease cleavage. Versions of NS3/4A-pVAX were constructed to encode proteins in which each amino acid of the shown sequence other than the alanine residues was substituted with an alanine residue. Each alanine residue was substituted with a glycine residue. The encoded polypeptides were analyzed for protease activity. The red color indicates the 16 mutations which resulted in a protein that lacked all protease activity. The dark blue color indicates the 3 mutations which resulted in a protein that exhibited greatly enhanced protease activity compared to wtNS3/4A.

[0124] FIGURE 22 shows the production of IFN- γ by splenocytes of mice immunized with various DNA and peptide antigens when primed with various antigen.

[0125] FIGURE 23 shows the level of IgE production by mice immunized with either recombinant birch or a DNA construct containing a birch-NS3/4A fusion gene.

[0126] FIGURE 24 shows the lysis of peptide loaded RMA-S cells.

[0127] FIGURE 25(a) shows the levels of anti-HBc IgG primed by three monthly injections with 100 μ g HBcAg-DNA im, 2 μ g HBcAg DNA transdermally by the gene gun with, or without, CPG as an adjuvant. Figure 25(b) shows the anti-HBc IgG subclass distribution after third immunization in the same groups of mice as in a. Figure 25(c) the adjuvanting effect of in vivo electroporation on the humoral responses after im injection of 0.5, 5, and 50 μ g of HBcAg DNA has been given. All data has been given as the mean end-point titres of the respective group.

[0128] FIGURE 26(a-n) show the efficiency of CTL priming by different modes of immunization, and doses with HBcAg DNA are shown as the percent specific lysis of HBcAg-, or NS3-, peptide loaded RMA-S cells.

[0129] FIGURE 27(a-h) shows the percent specific lysis of peptide loaded RMA-S cells. Figure 27(i) shows the percent HBcAg-specific CD8⁺ T cells by a direct ex-vivo quantification of HBcAg-specific CTLs using pentamer staining.

[0130] FIGURE 28(a-h) shows ELISpot assays of IFN γ producing HBcAg-specific T cells when HBcAg DNA was delivered by in vivo electroporation. FIGURE 28(i) shows the in vivo functionality of HBcAg-specific CTLs primed by high doses (2 x 100 μ g) of DNA given i.m.m without in vivo EP determined as clearance of HBcAg-expressing hepatocytes in transiently transgenic mice - Mice were given a hydrodynamic injection of

HBcAg DNA and 24 to 72 hours later levels were analyzed for the presence of HBcAg by immunoprecipitation followed by western blot. Lanes 1 to 8 indicate individual mice and controls are recombinant HBcAg and a 20kD size marker.

[0131] FIGURE 29(a-l) show the kinetics of induction of γ IFN producing HBcAg-specific CD8+ T cells in H-2b C57BL/6 (FIGURES 29(a and b) or CD4-/- (FIGURES 29(c and d)) knock-out mice as determined by ELISpot-assay. Pre-treatment with Th-peptide could not improve the poorly induced HBc-specific priming of CD8+ seen in mice immunized by gene gun (FIGURES 29(e to l)). To see if the priming of HBcAg-specific CD4+ T cells was a limiting factor for the priming of specific CTLs, mice were pre-treated with 100 μ g of a Th-peptide in IFA s.c. nine to 11 days before DNA immunization. HBcAg-specific γ IFN producing CD8+ and CD4+ cells were determined by ELISpot. Mice were divided into groups of five and received either 100 μ g pVAX1-HBc i.m. or 2 μ g pVAX1-HBc transdermally by gene gun. Mice received one (FIGURES 29(e to h)) or two immunizations (FIGURES 29 (i to l)) at weeks 0 and 4. Values are presented as number of μ IFN spot forming unit (SFU) per 10^6 cells.

[0132] FIGURE 30 shows the values of the ELISpot as number of γ IFN spot forming unit (SFU) per 10^6 cells. Codon optimization improves the ability of HBcAg DNA to induce specific IFN γ producing HBcAg-specific T cells at high doses when delivered by in vivo electroporation. C57BL/6 mice were primed once with 50, 5 or 0.5 μ g wtHBcAg DNA and HBcAg-specific γ IFN producing responses were determined 14 days later using ELISpot.

[0133] FIGURE 31 shows the percent HBcAg-specific CD8+ T cells.

[0134] FIGURE 32(a to f) show lytic HBcAg-specific CTLs at high doses. FIGURE 32(g) shows anti-HBc levels.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0135] Several of the embodiments described herein concern compositions and methods that are useful for generating, enhancing, or improving an immune response to an epitope of a target antigen. Disclosed herein are compositions relating to genetic constructs that include sequences from the Hepatitis C virus (HCV) and sequences encoding T-cell epitopes (TCEs), and methods of generating or enhancing an immune response using the

genetic constructs and polypeptides encoded by the genetic constructs. Several embodiments disclosed herein provide nucleic acids that encode chimeric HCV NS3/4A polypeptides or fragments thereof of at least 3 amino acids in length. For example, the NS3/4A sequence or a fragment thereof can comprise at least, equal to, greater than, or less than, or any number in between 3, 5, 10, 20, 50, 100, 150, 200, 250, 300, 350, 400, 500, 700, 1000, 1200, or 1500 consecutive amino acids of a natural or synthetic NS3/4A polypeptide (*e.g.*, a naturally occurring isotype or a codon-optimized or otherwise modified NS3/4A polypeptide). Exemplary NS3/4a sequences are disclosed in U.S. Patent No. 6,960,569, hereby expressly incorporated by reference in its entirety. Exemplary codon-optimized sequences are disclosed in U.S. Patent Application Publication No. 2003/0206919, hereby expressly incorporated by reference in its entirety, and in **SEQ ID NOs: 35 and 36**. That is, the nucleic acid encoding the NS3/4A sequence or a fragment thereof can comprise at least, equal to, greater than, less than, or any number in between 9, 15, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, or 2000 consecutive nucleotides of a nucleic acid sequence that encodes a natural or synthetic NS3/4A polypeptide. Many of these embodiments also include a nucleic acid that encodes at least one TCE located within or flanking (*e.g.*, juxtaposed to) the NS3/4A encoding fragment, such that the TCE is in a non-naturally occurring position. The encoded polypeptide retains catalytic activity (*i.e.*, NS3 protease and/or NS3 helicase activity). Embodiments disclosed herein also provide chimeric NS3/4A polypeptides or fragments thereof of at least 3 amino acids in length, which include a TCE within or flanking (*e.g.*, juxtaposed to) the NS3/4A sequences, such that the TCE is in a non-naturally-occurring position.

[0136] Generally, the generation, enhancement, or improvement of an immune response refers to an induction of a humoral (antibody) response and/or a cellular response. Most simply, an increase in the amount of antigen-specific antibodies (*e.g.*, total IgG) can be seen by utilizing one or more of the embodiments described herein. Enhancement of an immune response refers to any statistically significant change in the level of one or more immune cells (T cells, B cells, antigen-presenting cells, dendritic cells and the like) or in the activity of one or more of these immune cells (cytotoxic T lymphocyte (CTL) activity, helper T lymphocyte (HTL) activity, cytokine secretion, change in profile of cytokine secretion). The skilled artisan will readily appreciate that several methods for establishing whether an

immune response is generated, enhanced, or improved are available. A variety of methods for detecting the presence and levels of an immune response are available, for example. (*See, e.g.*, Current Protocols in Immunology, Ed: John E. Coligan, et al. (2001) John Wiley & Sons, NY, NY; Current Protocols in Molecular Biology, (2001), Greene Publ. Assoc. Inc. & John Wiley & Sons, NY, NY; Ausubel et al. (2001) Current Protocols in Molecular Biology, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., NY, N.Y.; Sambrook et al. (1989) Molecular Cloning, Second Ed., Cold Spring Harbor Laboratory, Plainview, N.Y.); Maniatis et al. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, N.Y.; and elsewhere). Illustrative methods useful in this context include intracellular cytokine staining (ICS), ELISPOT, proliferation assays, cytotoxic T cell assays including chromium release or equivalent assays, and gene expression analysis using any number of polymerase chain reaction (PCR) or RT-PCR based assays. For example, the number of CD8⁺ T-cells specific for a particular antigen or TCE can be measured by flow cytometry. (*See, e.g.*, Frelin et al. (2004) Gene Therapy 11:522-533). CTL priming can also be measured *in vivo* by, for example, a tumor inhibition model, in which the ability of an animal (*e.g.*, mouse) to inhibit growth of tumors derived from tumor cells engineered to express the antigen of interest. *Id.*

[0137] In some embodiments, generation or enhancement of an immune response comprises an increase in target-specific CTL activity of between 1.5 and 5 fold in a subject that is provided a composition that comprises the nucleic acids or polypeptides disclosed herein (*e.g.*, in the context of a chimeric NS3/4A nucleic acid or polypeptide), wherein the TCE is derived from the target, as compared to the same TCE that is not provided in the context of the compositions disclosed herein. In some embodiments, an enhancement of an immune response comprises an increase in target-specific CTL activity of about 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 15, 16, 17, 18, 19, 20, or more fold in a subject that is provided a composition that comprises a nucleic acid or a polypeptide disclosed herein (*e.g.*, in the context of a chimeric NS3/4A nucleic acid or polypeptide), wherein the TCE is derived from the target, as compared to as compared to administration of the same TCE that is not provided in the context of the compositions disclosed herein.

[0138] In other embodiments, an alteration of an immune response comprises an increase in target-specific HTL activity, such as proliferation of helper T cells, of between

1.5 and 5 fold in a subject that is provided a composition that comprises a nucleic acid or polypeptide disclosed herein (*e.g.*, in the context of a chimeric NS3/4A nucleic acid or polypeptide), wherein the TCE is derived from the target, as compared to the same TCE that is not provided in the context of the compositions disclosed herein. In some embodiments, alteration of an immune response comprises an increase in target-specific HTL activity of about 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 15, 16, 17, 18, 19, 20, or more fold in a subject that is provided a composition that comprises a nucleic acid or polypeptide disclosed herein (*e.g.*, in the context of a chimeric NS3/4A nucleic acid or polypeptide), wherein the TCE is derived from the target, as compared to administration of the same TCE that is not provided in the context of the compositions disclosed herein. In this context, an enhancement in HTL activity may comprise an increase as described above, or decrease, in production of a particular cytokine, such as interferon-gamma (IFN γ), interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-7, IL-12, IL-15, tumor necrosis factor-alpha (TNF α), granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte -colony stimulating factor (G-CSF), or other cytokine. In this regard, generation or enhancement of an immune response may comprise a shift from a Th2 type response to a Th1 type response or in certain embodiments a shift from a Th1 type response, to a Th2 type response. In other embodiments, the generation or enhancement of an immune response may comprise the stimulation of a predominantly Th1 or a Th2 type response.

[0139] In still more embodiments, an increase in the amount of antibody specific for the antigen (*e.g.*, total IgG) is increased. Some embodiments, for example, generate an increase in heterologous target-specific antibody production of between 1.5, 2, 3, 4, or 5 fold in a subject that is provided a composition comprising the nucleic acids or polypeptides disclosed herein, (*e.g.*, in the context of a chimeric NS3/4A nucleic acid or polypeptide), wherein the TCE is derived from the target, as compared to the same TCE that is not present in the context of the compositions disclosed herein. In some embodiments, the increase in heterologous target-specific antibody production is about 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 15, 16, 17, 18, 19, 20, or more fold in a subject that is provided a composition that comprises a nucleic acid or polypeptide disclosed herein, (*e.g.*, in the context of a chimeric NS3/4A nucleic acid or polypeptide), wherein the

TCE is derived from the target, as compared to as compared to administration of the same TCE that is not present in the context of the compositions disclosed herein.

[0140] Generation or enhancement of a cellular immune response can also refer to the frequency of cytotoxic T lymphocytes (CTLs) specific for a desired antigen that are primed, or the rapidity of priming of cytotoxic T lymphocytes (CTLs) specific for a desired antigen, compared to the priming of CTLs specific for the desired epitope when the epitope is not presented in the context of the nucleic acids or peptides disclosed herein. The section below describes several of the NS3/4A sequences that can be used in the compositions and methods described herein.

HCV Sequences

[0141] Several embodiments described herein provide genetic constructs that contain HCV sequences from the NS3/NS4A region of HCV. The NS3/NS4A region of HCV has been studied extensively. NS3, due to its limited genetic variability and relatively large size (631 amino acids) has in itself been studied as an attractive target for generating immune responses against HCV. (See Bartenschlager R., et al. (1995) J. Virol. 67:3835-3844; Pang et al. (2002) EMBO J. 21:1168-1176). The fact that NS3 is a relatively large protein renders it less likely to exhibit genetic non-responder status at the T cell level. (See Frelin et al. (2003) Gene Therapy 10:689-699). Accordingly, it was contemplated that the NS3 region of HCV is useful in genetic constructs for generating or enhancing an immune response to an accompanied target antigen (*e.g.*, in constructs that encode a TCE derived from a pathogen).

[0142] The catalytic activity of NS3 is known to affect a host's ability to mount an immune response to HCV. (See, *e.g.*, Foy, et al. (2005), Proc. Nat. Acad. Sci. USA 102(8): 2986-2991; Meylan, et al. (2005) Nature 437(20)1167-1172; Li et al. (2005) Proc. Nat. Acad. Sci. USA 102(8): 2992-2997). Accordingly, embodiments described herein relate to genetic constructs encoding catalytically active NS3/4A polypeptide derivatives, or functional fragments thereof, as well as the polypeptides encoded by the genetic constructs. As used herein, the term "functional fragment" of a polypeptide refers to a variant of the polypeptide that is not full-length yet retains desired attributes, (*e.g.* NS3A protease and/or

helicase activity, NS4A co-factor activity, or immunogenicity) of the full-length native sequence.

[0143] The NS3 protein of HCV possesses both protease and helicase activity. (See Liu, D. et al., (2001) J. Mol. Biol. 314:543-561). In preferred embodiments, compositions disclosed herein include sequences that retain NS3 protease and/or helicase activity. In addition to cleaving the HCV polypeptide, NS3 protease cleaves host proteins that normally function to activate the host's innate immune response. (See, e.g., Foy, et al. (2005), Proc. Nat. Acad. Sci. USA 102(8): 2986-2991; Meylan, et al. (2005) Nature 437(20):1167-1172; Li et al. (2005) Proc. Nat. Acad. Sci. USA 102(8): 2992-2997). Specifically, NS3 has been shown to cleave the Toll-like receptor 3 adaptor protein TRIF as well as Cardif. *Id.* Accordingly, in some embodiments, the NS3/4A nucleic acid sequences encode a polypeptide that comprises an NS3 protease domain (e.g., a sequence that exhibits protease activity).

[0144] NS3 protease activity is localized within the first 181 amino acids of the of NS3/4A peptide. (See, Lin, C. et al., (1994) J. Virol. 68(12):8147-8157). The NS3 protease domain has a trypsin-like serine proteinase motif and a zinc binding site. (See, Love, R. (1996) Cell 87:331-342). Three residues, His57, Asp81 and Ser139 constitute a catalytic triad typical of the trypsin-like serine proteases that are strictly conserved in all HCV genotype sequences. Strict conservation of spacing and order of these residues is also seen. The active site also contains an oxyanion/stabilization loop. The zinc binding site of NS3 is located within amino acids Cys97, Cys99, Cys145 and His149. *Id.* The zinc binding site is more highly conserved than the active site and is responsible for stabilizing the structure of the active site. *Id.*

[0145] The crystal structure HCV NS3 with an NS4A polypeptide has been solved. (See Yao, et al. (1999), Structure 7:1353-1363). Thus, where the NS3 protease domain contains, for example, an alpha-helix or a beta-sheet structure, in some embodiments, variants or modified NS3/4A molecules comprise insertions of amino acids that maintain that specific structure. In addition to the structural information above, we describe herein experimental results in which each and every residue in the NS3 protease domain was systematically mutated and tested for protease activity, thus providing guidance in relation to NS3/4A variants, such as which amino acids in the NS3 protease domain are preferably

preserved in embodiments that retain NS3 protease activity, as well as positions along the protease domain that can tolerate insertions of TCEs and/or TCEs and linkers, as discussed in more detail below.

[0146] As used herein the phrase “NS3 protease domain” refers to sequences encoding the NS3 protease domain from any or all HCV genotypes or isotypes now known or discovered in the future. Nucleic acids encoding NS3 protease domains include any nucleic acid, taking into account the degeneracy of the genetic code that encodes an NS3 protease domain, and also including codon-optimized NS3 sequences and modified NS3 sequences derived from naturally-occurring NS3 nucleic acids. Non-limiting examples of NS3/4A nucleic acid sequences that can be used with the embodiments described herein include **SEQ ID NOs: 1, 35, and 572-808**. By way of example, NS3 helicase domains can comprise nucleic acid residues 1-551 of **SEQ ID NO:35**, or analogous residues in any NS3/4A nucleic acid. **SEQ ID NO: 35** is an exemplary codon-optimized sequence of a nucleic acid encoding an NS3/NS4A protein generated from an HCV isolate.

[0147] The NS3 helicase domain resides in the C terminal 450 amino acids of the protein. Yao et al. (1997) Nat. Struct. Biol. 4(6):463-467. The structure of the helicase domain by itself, in complex with single-stranded DNA, and in the bifunctional protease-helicase complexes with NS4A has been solved. (*Id.* and Kim et al. (1998), Structure 6:89-100). Previous studies have indicated that the protease domain of NS3 enhances the helicase activity of NS3. (*See*, Frick et al. (2004) J Biol Chem. 279(2):1269-1280). The available structural information above provide guidance as to the nature of NS3/4A variants, which include substitutions, insertions and deletions in the NS3 helicase domain that can be made without perturbing the catalytic activity of the helicase domain, for example in embodiments that retain NS3 helicase activity.

[0148] As used herein, the phrase “NS3 helicase domain” refers to sequences encoding an NS3 helicase domain from any or all HCV genotypes now known or discovered in the future. Nucleic acids encoding NS3 helicase domains include any nucleic acid, taking into account the degeneracy of the genetic code that encodes an NS3 polypeptide and also including codon-optimized NS3 helicase sequences and modified NS3 helicase sequences derived from naturally-occurring NS3 helicase nucleic acids. Non-limiting examples of NS3/4A nucleic acid sequences, including sequences of NS3 helicase domains, are **SEQ ID**

NOs: 1, 35, and 572-808. By way of example, NS3 helicase domains can comprise nucleic acid residues 218-1568 of **SEQ ID NO:35**, or analogous residues in any NS3/4A nucleic acid. **SEQ ID NO: 35** is an exemplary codon-optimized nucleic acid sequence of an NS3/NS4A peptide generated from an HCV isolate.

[0149] The NS4 polypeptide of HCV has been shown to increase the intracellular stability of NS3 and target NS3 to intracellular membranes, thereby potentially increasing the immunogenicity of NS3. (See, Wolk, B. et al. (2000). J. Virol. 74:2293-2304). We recently demonstrated that NS4A gene from HCV is an enhancer that increases transcription and immunogenicity of an associated gene or nucleic acid (e.g., NS3). (See, WO 04/048403, which designated the United States and was published in English, the disclosure of which is hereby expressly incorporated by reference in its entirety). The data illustrate that when HCV-1 NS3/4A was transfected into mammalian cells, *vis a vis* a eukaryotic expression vector, the expression level of NS3 was increased compared to the expression levels of NS3 alone (*i.e.*, without NS4A). Further, immunization with an NS3/NS4A construct was shown to prime NS3-specific CTLs, when the construct was provided either *i.m.* or transdermally. Accordingly, embodiments disclosed herein include sequences encoding an NS4A polypeptide, variant, or functional fragment thereof.

[0150] As used herein, the term “NS4A” refers to either nucleic acid or amino acid sequences of the NS4A region from any and all HCV genotypes now known or discovered in the future. Nucleic acids encoding NS4A include any nucleic acid, taking into account the degeneracy of the genetic code, that encodes an NS4A domain and also includes codon-optimized NS4A sequences and modified NS4A sequences derived from naturally-occurring NS4A nucleic acids. Non-limiting examples of NS3/4A nucleic acid sequences, including sequences of NS4 co-factor domains, are **SEQ ID NOs: 1, 35, 567-804**. By way of example, NS3 helicase domains can comprise nucleic acid residues 1569-2069 of **SEQ ID NO:35**, or analogous residues in any NS3/4A nucleic acid. **SEQ ID NO: 35** is an exemplary codon-optimized nucleic acid sequence of an NS3/NS4A peptide generated from an HCV isolate. **SEQ ID NO:36** is an exemplary codon-optimized amino acid sequence of an NS3/4A peptide generated from an HCV isolate.

[0151] Current listings of exemplary HCV nucleic acid and polypeptide sequences, including NS3/NS4A, are publicly available at the Los Alamos National

Laboratories world-wide web site. HCV NS3/4A nucleic acid sequences (including novel NS3/NS4A regions) can also be isolated from patients infected with HCV using the nucleic acids described herein. (*See also, Example 1*). RNA obtained from a patient infected with HCV can be reverse transcribed and the resultant cDNA can be amplified using PCR or another amplification technique. The primers are preferably obtained from the NS3/4A sequence of **SEQ. ID. NO.: 1**.

[0152] For a review of PCR technology, see *Molecular Cloning to Genetic Engineering* White, B.A. Ed. in *Methods in Molecular Biology* 67: Humana Press, Totowa (1997) and the publication entitled "PCR Methods and Applications" (1991, Cold Spring Harbor Laboratory Press). For amplification of mRNAs, it is within the scope of the invention to reverse transcribe mRNA into cDNA followed by PCR (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Patent No. 5,322,770. Another technique involves the use of Reverse Transcriptase Asymmetric Gap Ligase Chain Reaction (RT-AGLCR), as described by Marshall R.L. et al. (*PCR Methods and Applications* 4:80-84, 1994).

[0153] Briefly, RNA is isolated, following standard procedures. A reverse transcription reaction is performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment as a primer of first strand synthesis. The resulting RNA/DNA hybrid is then "tailed" with guanines using a standard terminal transferase reaction. The hybrid is then digested with RNase H, and second strand synthesis is primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment are easily isolated. For a review of cloning strategies which can be used see *e.g.*, Sambrook et al., 1989, *supra*.

[0154] In each of these amplification procedures, primers on either side of the sequence to be amplified are added to a suitably prepared nucleic acid sample along with dNTPs and a thermostable polymerase, such as Taq polymerase, Pfu polymerase, or Vent polymerase. The nucleic acid in the sample is denatured and the primers are specifically hybridized to complementary nucleic acid sequences in the sample. The hybridized primers are then extended. Thereafter, another cycle of denaturation, hybridization, and extension is initiated. The cycles are repeated multiple times to produce an amplified fragment containing the nucleic

acid sequence between the primer sites. PCR has further been described in several patents including US Patents 4,683,195, 4,683,202 and 4,965,188.

[0155] The primers are selected to be substantially complementary to a portion of the nucleic acid sequence of (SEQ. ID. NO.: 1) that is unique to this NS3/4A molecule, thereby allowing the sequences between the primers to be amplified. Preferably, primers can be any number between at least 16-20, 20-25, or 25-30 nucleotides in length. The formation of stable hybrids depends on the melting temperature (T_m) of the DNA. The T_m depends on the length of the primer, the ionic strength of the solution and the G+C content. The higher the G+C content of the primer, the higher is the melting temperature because G:C pairs are held by three H bonds whereas A:T pairs have only two. The G+C content of the amplification primers described herein preferably range between 10% and 75 %, more preferably between 35% and 60%, and most preferably between 40% and 55 %. The appropriate length for primers under a particular set of assay conditions can be empirically determined by one of skill in the art.

[0156] The spacing of the primers relates to the length of the segment to be amplified. In the context of the embodiments described herein, amplified segments carrying nucleic acid sequence encoding HCV peptides can range in size from at least about 25 bp to the entire length of the HCV genome. Amplification fragments from 25-1000 bp are typical, fragments from 50-1000 bp are preferred and fragments from 100-600 bp are highly preferred. It will be appreciated that amplification primers can be of any sequence that allows for specific amplification of the NS3/4A region and can, for example, include modifications such as restriction sites to facilitate cloning.

[0157] The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequences of an HCV peptide. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library. Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from an infected patient. In this manner, HCV gene products can be isolated using standard antibody screening techniques in conjunction with antibodies raised against the HCV gene product.

(For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor).

NS3/NS4A variant sequences

[0158] A novel nucleic acid and protein corresponding to the NS3/4A domain of HCV was cloned from a patient infected with HCV (**SEQ. ID. NO.: 1**). A Genbank search revealed that the cloned sequence had the greatest homology to HCV sequences but was only 93% homologous to the closest HCV relative (accession no AJ 278830). This novel peptide (**SEQ. ID. NO.: 2**) and fragments thereof (e.g., **SEQ. ID. NOs.: 14** and **15**) that are any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length), nucleic acids encoding these molecules, vectors having said nucleic acids, and cells having said vectors, nucleic acids, or peptides are embodiments of the invention. It was also discovered that both the NS3/4A gene (**SEQ. ID. NO.: 1**) and corresponding peptide (**SEQ. ID. NO.: 2**) were immunogenic *in vivo*.

[0159] In certain embodiments, the NS3/4A nucleic acids and polypeptides of the compositions and methods disclosed herein include variations in nucleotide and/or amino acid sequences, compared to native NS3/4A sequences and are referred to as NS3/4A variants. As used herein, the term "native" refers to naturally occurring HCV sequences (e.g., available HCV isotypes). Variants may include a substitution, deletion or insertion of one or more nucleotides, amino acids, or codons encoding the NS3/4A sequences of the chimeric NS3/4A polypeptides, which results in a change in the amino acid sequence of the NS3/4A polypeptide, as compared with the native sequence. Variants can be engineered, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934.

[0160] Mutants of the novel NS3/4A peptide were created. It was discovered that truncated mutants (e.g., **SEQ. ID. NOs.: 12** and **13**) and mutants that lack a proteolytic cleavage site (**SEQ. ID. NOs.: 3-11**), were also immunogenic *in vivo*. These novel peptides (**SEQ. ID. NOs.: 3-13**) and fragments thereof (e.g., **SEQ. ID. NOs.: 16-26**) that are any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length), nucleic acids encoding these molecules, vectors

having said nucleic acids, and cells having said vectors, nucleic acids, or peptides are also embodiments of the invention.

[0161] A codon-optimized nucleic acid encoding NS3/4a was also created and was found to be immunogenic. The nucleic acid of **SEQ. ID. NO.: 1** was analyzed for codon usage and the sequence was compared to the codons that are most commonly used in human cells. Because HCV is a human pathogen, it was unexpected to discover that the virus had not yet evolved to use codons that are most frequently found to encode human proteins (e.g., optimal human codons). A total of 435 nucleotides were replaced to generate the codon-optimized synthetic NS3/4A nucleic acid. The NS3/4A peptide encoded by the codon-optimized nucleic acid sequence (**SEQ. ID. NO.: 36**) was 98% homologous to HCV-1 and contained a total of 15 different amino acids.

[0162] The codon optimized nucleic acid (MSLF1 or coNS3/4A) (**SEQ. ID. NO.: 35**) was found to be more efficiently translated *in vitro* than the native NS3/4A and that mice immunized with the MSLF1 containing construct generated significantly more NS3/4A specific antibodies than mice immunized with a wild-type NS3/4A containing construct. Further, mice immunized with the MSLF1 containing construct were found to prime NS3-specific CTLs more effectively and exhibit better *in vivo* tumor inhibiting immune responses than mice immunized with wild-type NS3/4A containing constructs.

[0163] NS3/NS4A genes encoding polypeptides with alanine or glycine substitutions in the serine protease domain of NS3 (i.e., the first 181 amino acids) (**SEQ ID NO's: 40 through 220 and 1329-1339**) were found to have altered protease activity compared to the wtNS3/NS4A polypeptide.

[0164] The peptides and nucleic acids described above are useful as immunogens, which can be administered alone or in conjunction with an adjuvant. Preferred embodiments include compositions that comprise one or more of the nucleic acids and/or peptides described above with or without an adjuvant. That is, some of the compositions described herein are prepared with or without an adjuvant and comprise, consist, or consist essentially of a NS3/4A peptide (**SEQ. ID. NO.: 2** or **SEQ. ID. NO.: 36**) or fragments thereof that are any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length) (e.g., **SEQ. ID. NOs.: 14 and 15**) or a nucleic acid encoding one or more of these molecules (e.g., **SEQ. ID. NO.: 35**) or a fragment thereof

that is any number of consecutive nucleotides between at least 12-2112 (e.g., 12-15, 15-20, 20-30, 30-50, 50-100, 100-200, 200-500, 500-1000, 1000-1500, 1500-2079, or 1500-2112 consecutive nucleotides in length). Additional compositions are prepared with or without an adjuvant and comprise, consist, or consist essentially of one or more of the NS3/4A mutant peptides (**SEQ. ID. NOs.: 3-13**) and fragments thereof that are any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length). Some of the compositions described herein are prepared with or without an adjuvant and comprise, consist, or consist essentially of a mutant NS3/NS4A peptide (e.g., **SEQ ID NO's: 40 through 220 and 1329-1339**), or a nucleic acid encoding one or more of these molecules.

[0165] It was also discovered that compositions comprising ribavirin and an antigen (e.g., one or more of the previously described HCV peptides or nucleic acids) enhance and/or facilitate an animal's immune response to the antigen. That is, it was discovered that ribavirin is a very effective "adjuvant," which for the purposes of this disclosure, refers to a material that has the ability to enhance or facilitate an immune response to a particular antigen. The adjuvant activity of ribavirin was manifested by a significant increase in immune-mediated protection against the antigen, an increase in the titer of antibody raised to the antigen, and an increase in proliferative T cell responses.

[0166] Accordingly, compositions (e.g., vaccines and other medicaments) that comprise ribavirin and one or more of the peptides or nucleic acids described herein are embodiments of the invention. These compositions can vary according to the amount of ribavirin, the form of ribavirin, as well as the sequence of the HCV nucleic acid or peptide.

[0167] Embodiments of the invention also include methods of making and using the compositions above. Some methods involve the making of nucleic acids encoding NS3/4A, codon-optimized NS3/4A, mutant NS3/4A, fragments thereof that are any number of consecutive nucleotides between at least 9-100 (e.g., 9, 12, 15, 18, 21, 24, 27, 30, 50, 60, 75, 80, 90, or 100 consecutive nucleotides in length), peptides corresponding to said nucleic acids, constructs comprising said nucleic acids, and cells containing said compositions. Preferred methods, however, concern the making of vaccine compositions or immunogenic preparations that comprise, consist, or consist essentially of the newly discovered NS3/4A fragment, codon-optimized NS3/4A, or an NS3/4A mutant (e.g., a truncated mutant, a mutant

lacking a proteolytic cleavage site, or a mutant having altered protease activity), or a fragment thereof or a nucleic acid encoding one or more of these molecules, as described above. Preferred fragments for use with the methods described herein include **SEQ. ID. NOs.: 12-27** and fragments of **SEQ. ID. NO.: 35** that contain at least 30 consecutive nucleotides. The compositions described above can be made by providing an adjuvant (e.g., ribavirin), providing an HCV antigen (e.g., a peptide comprising an HCV antigen such as (**SEQ. ID. NOs.: 2-11, 36, or 40-220**) or a fragment thereof such as, **SEQ. ID. NOs.: 12-26** or a nucleic acid encoding one or more of said peptides), and mixing said adjuvant and said antigen so as to formulate a composition that can be used to enhance or facilitate an immune response in a subject to said antigen.

[0168] Methods of enhancing or promoting an immune response in an animal, including humans, to an antigen are also provided. Such methods can be practiced, for example, by identifying an animal in need of an immune response to HCV and providing said animal a composition comprising one or more of the nucleic acids or peptides above and an amount of adjuvant that is effective to enhance or facilitate an immune response to the antigen/epitope. In some embodiments, the antigen and the adjuvant are administered separately, instead of in a single mixture. Preferably, in this instance, the adjuvant is administered a short time before or a short time after administering the antigen. Preferred methods involve providing the animal in need with ribavirin and NS3/4A (e.g., **SEQ. ID. NO.: 2**), codon-optimized NS3/4A (e.g., **SEQ. ID. NO.: 36**), a mutant NS3/4A (e.g., **SEQ. ID. NOs.: 3-13 or 40-220**), a fragment thereof (e.g., **SEQ. ID. NOs.: 14-26**) containing any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length) or a nucleic acid encoding any one or more of said molecules.

[0169] Other embodiments concern methods of treating and preventing HCV infection. By one approach, an immunogen comprising one or more of the HCV nucleic acids or peptides described herein are used to prepare a medicament for the treatment and/or prevention of HCV infection. By another approach, an individual in need of a medicament that prevents and/or treats HCV infection is identified and said individual is provided a medicament comprising ribavirin and an HCV antigen such as NS3/4A (e.g., **SEQ. ID. NO.: 2**), codon-optimized NS3/4A (e.g., **SEQ. ID. NO.: 36**), or a mutant NS3/4A (e.g., **SEQ. ID.**

NOs.: 3-13 or **40-220**), a fragment thereof (e.g., **SEQ. ID. NOs.: 14-26**) containing any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length) or a nucleic acid encoding any one or more of these molecules.

[0170] The section below discusses the discovery of the novel NS3/4A gene, the codon-optimized NS3/4A gene, the creation of the NS3/4A mutants, and the characterization of the nucleic acids and peptides corresponding thereto.

NS3/4A, NS3/4A mutants, and Codon-Optimized NS3/4A

[0171] A novel nucleic acid and protein corresponding to the NS3/4A domain of HCV was cloned from a patient infected with HCV (**SEQ. ID. NOs.: 1** and **2**). A Genebank search revealed that the cloned sequence had the greatest homology to HCV sequences but was only 93% homologous to the closest HCV relative (accession no AJ 278830). A truncated mutant of the novel NS3/4A peptide and NS3/4A mutants, which lack a proteolytic cleavage site, (as well as corresponding nucleic acids) were also created. Further, a human codon-optimized NS3/4A nucleic acid and peptide were created. It was discovered that these novel peptides and nucleic acids encoding said peptides were potent immunogens that can be mixed with adjuvants so as to make a composition that induces a recipient to provide an immune response to HCV. The cloning of the novel NS3/4A gene and the creation of the various NS3/4A mutants and codon optimized NS3/4A gene are described in the following example.

EXAMPLE 1

[0172] The NS3/4A sequence was amplified from the serum of an HCV-infected patient (HCV genotype 1a) using the Polymerase Chain Reaction (PCR). Total RNA was extracted from serum, and cDNA synthesis and PCR were performed according to standard protocols (Chen M et al., *J. Med. Virol.* 43:223-226 (1995)). The cDNA synthesis was initiated using the antisense primer "NS4KR" (5'-CCG TCT AGA TCA GCA CTC TTC CAT TTC ATC-3' (**SEQ. ID. NO.: 28**)). From this cDNA, a 2079 base pair DNA fragment of HCV, corresponding to amino acids 1007 to 1711, which encompasses the NS3 and NS4A genes, was amplified. A high fidelity polymerase (Expand High Fidelity PCR, Boehringer-Mannheim, Mannheim, Germany) was used with the "NS3KF" primer (5'-CCT GAA TTC ATG GCG CCT ATC ACG GCC TAT-3' (**SEQ. ID. NO.: 29**) and the NS4KR primer. The

NS3KF primer contained a *EcoRI* restriction enzyme cleavage site and a start codon and the primer NS4KR contained a *XbaI* restriction enzyme cleavage site and a stop codon.

[0173] The amplified fragment was then sequenced (SEQ. ID. NO.: 1). Sequence comparison analysis revealed that the gene fragment was amplified from a viral strain of genotype 1a. A computerized BLAST search against the Genbank database using the NCBI website revealed that the closest HCV homologue was 93% identical in nucleotide sequence.

[0174] The amplified DNA fragment was then digested with *EcoRI* and *XbaI*, and was inserted into a pcDNA3.1/His plasmid (Invitrogen) digested with the same enzymes. The NS3/4A-pcDNA3.1 plasmid was then digested with *EcoRI* and *XbaI* and the insert was purified using the QiaQuick kit (Qiagen, Hamburg, Germany) and was ligated to a *EcoRI/XbaI* digested pVAX vector (Invitrogen) so as to generate the NS3/4A-pVAX plasmid.

[0175] The rNS3 truncated mutant was obtained by deleting NS4A sequence from the NS3/4A DNA. Accordingly, the NS3 gene sequence of NS3/4A-pVAX was PCR amplified using the primers NS3KF and 3'*NotI* (5'-CCA CGC GGC CGC GAC GAC CTA CAG-3' (SEQ. ID. NO.: 30)) containing *EcoRI* and *NotI* restriction sites, respectively. The NS3 fragment (1850 bp) was then ligated to a *EcoRI* and *NotI* digested pVAX plasmid to generate the NS3-pVAX vector. Plasmids were grown in BL21 *E.coli* cells. The plasmids were sequenced and were verified by restriction cleavage and the results were as to be expected based on the original sequence.

[0176] In some embodiments, nucleic acid sequences comprising, consisting essentially of, or consisting of sequences encoding TCEs are inserted within or flanking (e.g., juxtaposed to) the NS3/4A-encoding sequence described herein. In some embodiments, a linker or adjuvant sequence is also, optionally, inserted within or flanking (e.g., juxtaposed to) an NS3/4A native or variant sequence, or a native or variant TCE sequence. For example, the chimeric NS3/4A polypeptide encoded by the nucleic acids above can include sequences encoding a TCE, or a TCE flanked on one or both sides by linkers and/or adjuvant sequences, inserted between any two contiguous amino acids between amino acids 1 686 of a variant NS3/4A polypeptide (e.g., SEQ ID NO: 36). For example, in preferred embodiments, the

chimeric NS3/4A polypeptide encoded by the nucleic acids above can include sequences encoding a TCE, or a TCE flanked on one or both sides by linkers and/or adjuvant sequences, inserted between amino acids 453-513 of **SEQ ID NO:36**, or in an analogous position in any NS3/4A polypeptide. Embodiments also relate to the polypeptides encoded by said nucleic acids.

[0177] Accordingly, in some embodiments a nucleic acid encoding a TCE or a TCE and a linker(s) is inserted between the codons of an NS3/4A-encoding nucleic acid sequence. For example, in some embodiments, a nucleic acid encoding a TCE or a TCE and a linker(s) and/or an adjuvant sequence is inserted between any two contiguous nucleotides between nucleotides 3 and 2059 of an NS3/4A nucleic acid sequence such as **SEQ ID NO: 1**, or between any of the codons of a nucleic acid sequence encoding an NS3/4A variant (*e.g.*, **SEQ ID NO: 35**). For example, in some embodiments, a nucleic acid encoding a TCE or a TCE and a linker(s) and/or an adjuvant sequence is inserted between nucleotides 1370 and 1548 of **SEQ ID NO:35**, or in an analogous position in any NS3/4A nucleic acid. Embodiments also relate to polypeptides encoded by said nucleic acids.

[0178] In some embodiments, the nucleic acid sequences encoding the TCE or TCE and linker and/or adjuvant sequence portion of the chimeric NS3/4A polypeptide can be juxtaposed to the 5' end of the NS3/4A sequences, and encoding a chimeric NS3/4A polypeptide with a TCE or TCE and linker(s) and/or adjuvant sequence on the N-terminal end of the NS3/4A polypeptide. In some embodiments, the nucleic acid sequences encoding the TCE or TCE and linker(s) and/or adjuvant sequence polypeptide can be flanking (*e.g.*, juxtaposed to) the 3' end of the NS3/4A sequences and encode a chimeric NS3/4A polypeptides with a TCE or TCE and linker(s) and/or adjuvant sequence on the C-terminal end of the NS3/4A polypeptide. In embodiments in which the chimeric NS3/4A polypeptide comprises more than one TCE or TCE and linker(s) and/or adjuvant sequence, the nucleic acids encoding the TCEs can be located different positions relative to the nucleic acids encoding the NS3/4A sequences (*i.e.*, 5', within, or 3') and relative to each other. Optionally, NS3/4A variants include a substitution of at least one amino acid with any other amino acid in one or more of the domains of a different NS3/NS4A sequences. Embodiments also relate to polypeptides encoded by said nucleic acid sequences.

[0179] The skilled artisan will readily appreciate that a variety of techniques can be used to generate variants, such as the generation of insertions of desired sequences (*e.g.*, TCEs and linkers) within NS3/4A nucleic acid and polypeptide sequences described herein. For example, overlapping PCR can be used to generate desired substitutions or insertions (*e.g.*, a nucleic acid encoding a TCE, and/or linker sequences) within the NS3/4A sequences, or at the 3' or 5' ends of the NS3/4A sequences. (*See, e.g.*, Ho et al. (1989), *Gene* 77(1):51-9). Several commercially available kits are also available to facilitate site-directed mutagenesis, to facilitate the generation of NS3/4A variants, such as the recombinant nucleic acids and encoded polypeptides disclosed herein. An exemplary commercially available kit useful for generating chimeric NS3/4A polypeptides and chimeric NS3/4A polypeptide variants is the QUICKCHANGE® site directed mutagenesis kit (Stratagene, La Jolla, CA).

[0180] In preferred embodiments, the catalytic activity (*e.g.*, the protease or helicase activity) of a chimeric NS3/4A or chimeric NS3/4A variant may be enhanced or unchanged, relative to the native polypeptide, or may be diminished by less than 50%, and preferably less than 20% relative to the native polypeptide. In some embodiments the protease activity of an NS3/4A chimeric polypeptide or chimeric polypeptide variant is diminished by less than 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. In some embodiments the protease activity of an NS3/4A variant may be enhanced by at least 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. Exemplary NS3/4A variants with altered protease activity are discussed in further detail herein.

[0181] In preferred embodiments, the NS3/4A chimeric polypeptide retains protease activity. Accordingly, in some embodiments, the nucleic acids encoding the chimeric NS3/4A polypeptide encode or the chimeric NS3/4A polypeptides comprise the native amino acid sequence at the following positions of the NS3/4A sequence: Leu44, Ile48, Trp53, His57, Asp81, Trp85, Ala91, Leu94, Cys97, Cys99, Leu106, Thr108, Arg123, Gly124, Leu126, Ser139, Gly140, Leu143, Leu144, Cys145, His149, Ile153, Phe169, and Leu175. That is, the aforementioned residues are unchanged in some embodiments or, in some embodiments, the nucleic acids encoding TCEs or TCEs and linkers and/or adjuvant sequence are not substituted for, inserted within, or inserted at positions adjacent to nucleic

acid sequences encoding the following amino acids of NS3/4A sequences: Leu44, Ile48, Trp53, His57, Asp81, Trp85, Ala91, Leu94, Cys97, Cys99, Leu106, Thr108, Arg123, Gly124, Leu126, Ser139, Gly140, Leu143, Leu144, Cys145, His149, Ile153, Phe169, and Leu175.

[0182] In some embodiments, the chimeric NS3/4A variants exhibit enhanced protease activity. Embodiments disclosed herein provide NS3/4A chimeric polypeptides including one or more of the following amino acid substitutions in the NS3/4A sequence: Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly, Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, or Thr177Ala. That is, in some embodiments one or more of the following amino acid substitutions are included: Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly, Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, or Thr177Ala.

[0183] In some embodiments, NS3/4A variant sequences used in the embodiments disclosed herein lack a proteolytic cleavage site, such as **SEQ. ID. NOs.: 14** and **16-26**. In some embodiments, fragments of the NS3/4A variant sequences containing any number of consecutive amino acids between at least 3-300 amino acids (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, or 300 consecutive amino acids) of any one of **SEQ. ID. NOs.: 14** and **16-26** are used in the embodiments disclosed herein. Other exemplary NS3/4A variants with altered protease activity may generally be identified by modifying one or more of the above nucleic acid or polypeptide sequences and evaluating the protease activity of the variant, as discussed in further detail in **Example 1**.

[0184] In some embodiments, the helicase activity of a NS3/4A variant is diminished by less than 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. In some embodiments the helicase activity of an NS3/4A variant may be enhanced by at least 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the helicase activity as described herein

[0185] Guidance in determining the identity of amino acids that may affect NS3 helicase activity can be found by comparing the sequence of the NS3 helicase domain with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. An exemplary assay for testing variants for helicase activity is discussed in Artsaenko O, et al., (2003) J Gen Virol. 2003 84(Pt 9):2323-32, and Zhang et al., (2005) J Virol. 79(14):8687-97; and Kyono et al., (2004) J Biochem (Tokyo) 135(2):245-52.

[0186] Optionally, chimeric NS3/4A variants can encode or comprise amino acid substitutions, wherein one amino acid is substituted with another amino acid having similar structural and/or chemical properties (e.g., conservative amino acid replacements). A list of conservative amino acid substitutions can be found in **Table 1**.

TABLE 1

<u>Original Residue</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro; ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	leu
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

[0187] In some embodiments, variant NS3/4A sequences are engineered or optimized for codons most frequently used in humans. The nucleic acid sequence of an exemplary codon-optimized NS3/4A nucleic acid sequence (coNS3/4A) is provided in **SEQ. ID. NO.: 35**. The peptide encoded by said nucleic acid sequence is provided in **SEQ. ID. NO.: 36**. The skilled artisan will appreciate, however, that any HCV NS3/4A sequences disclosed herein or discovered in the future can be used to generate codon-optimized variants and that all codon-optimized variants are within the scope of the present invention.

[0188] The nucleic acid and corresponding NS3/4A peptide (**SEQ ID NOs: 35** and **36**) do not correspond to any known HCV sequence or genome. The codon-optimized NS3/4A encoding nucleic acid was found to be only 79% homologous, within the region of nucleotide positions 3417-5475, to HCV-1 and contained a total of 433 different nucleotides. The NS3/4A peptide encoded by the codon-optimized nucleic acid sequence is only 98% homologous to HCV-1 and contained a total of 15 different amino acids. As demonstrated in **Example 2**, below, the codon optimized nucleic acid was found to generate a higher expression level of NS3 and was found to be more immunogenic, with respect to both humoral and cellular responses, as compared to the native NS3/4A gene from which it was derived. Accordingly, in preferred embodiments, the NS3/4A nucleic acid sequences encoding, or the encoded polypeptide sequences of the NS3/4A chimeric polypeptides comprise codon-optimized nucleic acid and polypeptide sequences of native HCV sequences. For example, in some embodiments, the NS3/4A nucleic acid sequences or encoded polypeptide sequences of an NS3/4A chimeric polypeptides comprises **SEQ ID NO: 35** or **SEQ ID NO: 36**, or fragments thereof, or variants thereof and TCEs and/or linker sequences.

EXAMPLE 2

[0189] The sequence of the unique NS3/4A gene described in Example 1 (**SEQ. ID. NO.: 1**) was analyzed for codon usage with respect to the most commonly used codons in human cells. A total of 435 nucleotides were replaced to optimize codon usage for human cells. The sequence was sent to Retrogen Inc. (6645 Nancy Ridge Drive, San Diego, CA 92121) and they were provided with instructions to generate a full-length synthetic codon optimized NS3/4A gene. The codon optimized NS3/4A gene had a sequence homology of

79% within the region between nucleotide positions 3417-5475 of the HCV-1 reference strain. A total of 433 nucleotides differed. On an amino acid level, the homology with the HCV-1 strain was 98% and a total of 15 amino acids differed.

[0190] The full length codon optimized 2.1 kb DNA fragment of the HCV corresponding to the amino acids 1007 to 1711 encompassing the NS3 and NS4A NS3/4A gene fragment was amplified by the polymerase chain reaction (PCR) using high fidelity polymerase (Expand High Fidelity PCR, Boehringer-Mannheim, Mannheim, Germany). The amplicon was then inserted into a Bam HI and Xba I digested pVAX vector (Invitrogen, San Diego), which generated the MSLF1-pVAX (coNS3/4A-pVAX) plasmid. All expression constructs were sequenced. Plasmids were grown in competent BL21 E. Coli. The plasmid DNA used for in vivo injection was purified using Qiagen DNA purification columns, according to the manufacturers instructions (Qiagen GmbH, Hilden, FRG). The concentration of the resulting plasmid DNA was determined spectrophotometrically (Dynaquant, Pharmacia Biotech, Uppsala, Sweden) and the purified DNA was dissolved in sterile phosphate buffer saline (PBS) at concentrations of 1 mg/ml.

[0191] The expression of NS3 and NS3/4A proteins from the wtNS3/4A (wild-type NS3/4A) and coNS3/4A plasmids, were analyzed by an in vitro transcription and translation assay. The assay showed that the proteins could be correctly translated from the plasmids and that the coNS3/4A plasmid gave detectable NS3 and NS3/4A bands at a higher plasmid dilution as compared to the wtNS3/4A plasmid. This result provided strong evidence that the in vitro translation from the coNS3/4A plasmid is more effective than wtNS3/4A. To compare the expression levels more precisely, HepG2 cells were transiently transfected with the wtNS3/4A and the coNS3/4A plasmids. These experiments revealed that the coNS3/4A plasmid generated 11-fold higher expression levels of the NS3 protein when compared to the wtNS3/4A plasmid, as determined by densitometry and a standard curve of recombinant NS3. Since the wtNS3/4A and the coNS3/4A plasmids are identical in size it is unlikely that there would be any major differences in transfections efficiencies between the plasmids. Staining of coNS3/4A plasmid transfected, and SFV infected, BHK cells revealed a similar perinuclear and cytoplasmic distribution of the NS3 as previously observed, confirming an unchanged subcellular localization.

[0192] **TABLE 2** describes the sequence of the proteolytic cleavage site of NS3/4A, referred to as the breakpoint between NS3 and NS4A. This wild-type breakpoint sequence was mutated in many different ways so as to generate several different NS3/4A breakpoint mutants. **TABLE 2** also identifies these mutant breakpoint sequences. The fragments listed in **TABLE 2** are preferred immunogens that can be incorporated with or without an adjuvant (e.g., ribavirin) into a composition for administration to an animal so as to induce an immune response in said animal to HCV.

TABLE 2

Plasmid	Deduced amino acid sequence	SEQ ID
NS3/4A-pVAX	TKYMTCMSADLEVVT <u>ST</u> WVLVGGVL	14
NS3/4A-TGT-pVAX	TKYMTCMSADLEVVT <u>GT</u> WVLVGGVL	16
NS3/4A-RGT-pVAX	TKYMTCMSADLEVVR <u>GT</u> WVLVGGVL	17
NS3/4A-TPT-pVAX	TKYMTCMSADLEVVT <u>PT</u> WVLVGGVL	18
NS3/4A-RPT-pVAX	TKYMTCMSADLEVVR <u>PT</u> WVLVGGVL	19
NS3/4A-RPA-pVAX	TKYMTCMSADLEVVR <u>PA</u> WVLVGGVL	20
NS3/4A-CST-pVAX	TKYMTCMSADLEVVC <u>ST</u> WVLVGGVL	21
NS3/4A-CCST-pVAX	TKYMTCMSADLEVCC <u>ST</u> WVLVGGVL	22
NS3/4A-SSST-pVAX	TKYMTCMSADLEVSS <u>ST</u> WVLVGGVL	23
NS3/4A-SSSSCST-pVAX	TKYMTCMSADSSSSC <u>ST</u> WVLVGGVL	24
NS3A/4A-VVVVTST-pVAX	TKYMTCMSADVVVV <u>TST</u> WVLVGGVL	25
NS5-pVAX	ASEDVVCC <u>SM</u> SYTWTG	27
NS5A/B-pVAX	SSEDVVCC <u>SM</u> WVLVGGVL	26

*The wild type sequence for the NS3/4A fragment is NS3/4A-pVAX. The NS3/4A breakpoint is identified by underline, wherein the P1 position corresponds to the first Thr (T) and the P1' position corresponds to the next following amino acid the NS3/4A-pVAX sequence. In the wild type NS3/4A sequence the NS3 protease cleaves between the P1 and P1' positions.

[0193] To change the proteolytic cleavage site between NS3 and NS4A, the NS3/4A-pVAX plasmid was mutagenized using the QUICKCHANGE™ mutagenesis kit (Stratagene), following the manufacturer's recommendations. To generate the "TPT"

mutation, for example, the plasmid was amplified using the primers 5'-CTGGAGGTCGTCACGCCTACCTGGGTGCTCGTT-3' (SEQ. ID. NO.: 31) and 5'-ACCGAGCACCCAGGTAGGCGTGACGACCTCCAG-3' (SEQ. ID. NO.: 32) resulting in NS3/4A-TPT-pVAX. To generate the "RGT" mutation, for example, the plasmid was amplified using the primers 5'-CTGGAGGTCGTCCGCGGTACCTGGGTGCTCGTT-3' (SEQ. ID. NO.: 33) and 5'-ACCGAGCACCCAGGTACC-GCGGACGACCTCCAG-3' (SEQ. ID. NO.: 34) resulting in NS3/4A-RGT-pVAX. All mutagenized constructs were sequenced to verify that the mutations had been correctly made. Plasmids were grown in competent BL21 *E. coli*.

[0194] On an amino acid level, the homology with the HCV-1 strain was 98% and a total of 15 amino acids differed. The nucleic acid sequence of the codon-optimized NS3/4a is provided in **SEQ ID NO: 35**, whereas the peptide encoded by said nucleic acid sequence is provided in **SEQ ID NO:36**. The full length codon optimized 2.1 kb DNA fragment of the HCV corresponding to the amino acids 1007 to 1711 encompassing the NS3 and NS4A NS3/4A gene fragment was amplified by the polymerase chain reaction (PCR) using high fidelity polymerase (Expand High Fidelity PCR, Boehringer-Mannheim, Mannheim, Germany). The amplicon was then inserted into a *Bam* *HI* and *Xba* *I* digested pVAX vector (Invitrogen, San Diego), which generated the MSLF1-pVAX (coNS3/4A-pVAX) plasmid. All expression constructs were sequenced. Plasmids were grown in competent BL21 *E. Coli*. The plasmid DNA used for *in vivo* injection was purified using Qiagen DNA purification columns, according to the manufacturers instructions (Qiagen GmbH, Hilden, FRG). The concentration of the resulting plasmid DNA was determined spectrophotometrically (Dynaquant, Pharmacia Biotech, Uppsala, Sweden) and the purified DNA was dissolved in sterile phosphate buffer saline (PBS) at concentrations of 1 mg/ml.

[0195] The expression of NS3 and NS3/4A proteins from the wtNS3/4A (wild-type NS3/4A) and coNS3/4A plasmids, were analyzed by an *in vitro* transcription and translation assay. The assay showed that the proteins could be correctly translated from the plasmids and that the coNS3/4A plasmid gave detectable NS3 and NS3/4A bands at a higher plasmid dilution as compared to the wtNS3/4A plasmid. This result provided strong evidence that the *in vitro* translation from the coNS3/4A plasmid is more effective than wtNS3/4A. To compare the expression levels more precisely, HepG2 cells were transiently

transfected with the wtNS3/4A and the coNS3/4A plasmids. These experiments revealed that the coNS3/4A plasmid generated 11-fold higher expression levels of the NS3 protein when compared to the wtNS3/4A plasmid, as determined by densitometry and a standard curve of recombinant NS3. Since the wtNS3/4A and the coNS3/4A plasmids are identical in size it is unlikely that there would be any major differences in transfections efficiencies between the plasmids. Staining of coNS3/4A plasmid transfected, and SFV infected, BHK cells revealed a similar perinuclear and cytoplasmic distribution of the NS3 as previously observed, confirming an unchanged subcellular localization.

[0196] Several nucleic acid embodiments include nucleotides encoding the HCV peptides described herein (**SEQ. ID. NOs.: 2-11** or **SEQ. ID. NO.: 36**) or a fragment thereof (e.g., **SEQ. ID. NOs.: 14** and **15**) containing any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids in length). Some embodiments for example, include genomic DNA, RNA, and cDNA encoding these HCV peptides. The HCV nucleotide embodiments not only include the DNA sequences shown in the sequence listing (e.g., **SEQ. ID. NO.: 1** or **SEQ. ID. NO.: 35**) but also include nucleotide sequences encoding the amino acid sequences shown in the sequence listing (e.g., **SEQ. ID. NOs.: 2-11** or **SEQ. ID. NO.: 36**) and any nucleotide sequence that hybridizes to the DNA sequences shown in the sequence listing under stringent conditions (e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7.0% sodium dodecyl sulfate (SDS), 1 mM EDTA at 50°C) and washing in 0.2 X SSC/0.2% SDS at 50°C and any nucleotide sequence that hybridizes to the DNA sequences that encode an amino acid sequence provided in the sequence listing (**SEQ. ID. NOs.: 2-11** or **SEQ. ID. NO.: 36**) under less stringent conditions (e.g., hybridization in 0.5 M NaHPO₄, 7.0% sodium dodecyl sulfate (SDS), 1 mM EDTA at 37°C and washing in 0.2X SSC/0.2% SDS at 37°C).

[0197] The nucleic acid embodiments of the invention also include fragments, modifications, derivatives, and variants of the sequences described above. Desired embodiments, for example, include nucleic acids having at least 25 consecutive bases of one of the novel HCV sequences or a sequence complementary thereto and preferred fragments include at least 25 consecutive bases of a nucleic acid encoding the NS3/4A molecule of **SEQ. ID. NO.: 2** or **SEQ. ID. NO.: 36** or a mutant NS3/4A molecule of **SEQ. ID. NOs.: 3-13** or a sequence complementary thereto.

[0198] In this regard, the nucleic acid embodiments described herein can have any number of consecutive nucleotides between about 12 to approximately 2112 consecutive nucleotides of **SEQ. ID. NO.: 1** or **SEQ. ID. NO.: 35**. Some DNA fragments, for example, include nucleic acids having at least 12-15, 15-20, 20-30, 30-50, 50-100, 100-200, 200-500, 500-1000, 1000-1500, 1500-2079, or 1500-2112 consecutive nucleotides of **SEQ. ID. NO.: 1** or **SEQ. ID. NO.: 35** or a complement thereof. These nucleic acid embodiments can also be altered by substitution, addition, or deletion so long as the alteration does not significantly affect the structure or function (e.g., ability to serve as an immunogen) of the HCV nucleic acid. Due to the degeneracy of nucleotide coding sequences, for example, other DNA sequences that encode substantially the same HCV amino acid sequence as depicted in **SEQ. ID. NOs.: 2-13** or **SEQ. ID. NO.: 36** can be used in some embodiments. These include, but are not limited to, nucleic acid sequences encoding all or portions of HCV peptides (**SEQ. ID. NOs.: 2-13**) or nucleic acids that complement all or part of this sequence that have been altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change, or a functionally non-equivalent amino acid residue within the sequence, thus producing a detectable change. Accordingly, the nucleic acid embodiments of the invention are said to be comprising, consisting of, or consisting essentially of nucleic acids encoding any one of **SEQ. ID. NOs.: 2-27** or **SEQ. ID. NO.: 36** in light of the modifications above.

[0199] In some embodiments, the HCV NS3/4A sequences comprise, consist essentially of, or consist of NS3 and or NS4A sequences that encode fragments, or functional fragments of the full-length NS3/4A polypeptide. Such fragments may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full length native protein. In preferred embodiments, NS3/4A fragments lack amino acid residues that are not essential for the catalytic activity of the NS3 polypeptide. For example, in some embodiments the NS3/4A sequences can comprise, consist, or consist essentially of fragments of any of **SEQ ID NOs: 1, 35, and 572-808** encoding any number of consecutive amino acids (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, or 300 consecutive amino acids). The section below describes several antigens that can be used in the compositions and methods described herein.

Epitopes

[0200] The chimeric NS3/4A nucleic acids and polypeptides disclosed herein include sequences comprising antigens, such as TCEs, positioned at non-naturally occurring locations, either within and/or flanking (e.g., juxtaposed to) NS3/4A sequences. As used herein, the term epitope refers to a set of amino acid residues that are involved in recognition by a particular immunoglobulin (*i.e.*, a B cell epitope or “BCE”) or in the context of TCE, those residues necessary for recognition by T cell receptor proteins and/or Major Histocompatibility Complex (MHC) receptors. In an immune system setting, *in vivo* or *in vitro*, an epitope is the collective features of a molecule, such as primary, secondary, and tertiary peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor, or HLA molecule. TCEs are recognized by either CD4+ T cells, or helper T lymphocytes (“HTLs”) or CD8+ T cells, or cytotoxic T lymphocytes (“CTLs”).

[0201] TCEs generally comprise a chain of at least four amino acid residues, preferably at least six, more preferably eight to ten, sometimes eleven to fourteen residues, and usually fewer than about thirty residues, more usually fewer than about twenty-five, and preferably fewer than fifteen, e.g., eight to fourteen amino acid residues derived from selected epitopic regions of the target antigen(s). It is to be appreciated, however, that TCE nucleic acids, or TCE amino acid sequences can refer to nucleic acids encoding or protein or peptide molecules, larger than and comprising an epitope of the invention are still within the scope of the invention. For example, nucleic acid and polypeptide sequences of full length proteins that contain at least one TCE, that are capable of producing an immune response are contemplated for use in some embodiments.

[0202] In some aspects, the TCE are nucleic acids encoding peptides wherein said nucleic acids are, are at least, are at least about, are less than, or are less than about 3 nucleotides, 4 nucleotides, 5 nucleotides, 6 nucleotides, 7 nucleotides, 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, 30 nucleotides, 31 nucleotides, 32 nucleotides, 33 nucleotides, 34 nucleotides, 35 nucleotides, 36 nucleotides,

37 nucleotides, 38 nucleotides, 39 nucleotides, 40 nucleotides, 41 nucleotides, 42 nucleotides, 43 nucleotides, 44 nucleotides, 45 nucleotides, 46 nucleotides, 47 nucleotides, 48 nucleotides, 49 nucleotides, 50 nucleotides, 55 nucleotides, 60 nucleotides, 65 nucleotides, 70 nucleotides, 75 nucleotides, 80 nucleotides, 85 nucleotides, 90 nucleotides, 95 nucleotides, 100 nucleotides, 110 nucleotides, 120 nucleotides, 130 nucleotides, 140 nucleotides, 150 nucleotides, 160 nucleotides, 170 nucleotides, 180 nucleotides, 190 nucleotides, 200 nucleotides, 250 nucleotides, 300 nucleotides, 350 nucleotides, 400 nucleotides, 450 nucleotides, 500 nucleotides, 550 nucleotides, 600 nucleotides, 650 nucleotides, 700 nucleotides, 750 nucleotides, 800 nucleotides, 850 nucleotides, 900 nucleotides, 950 nucleotides, 1000 nucleotides, 1100 nucleotides, 1200 nucleotides, 1300 nucleotides, 1400 nucleotides, 1500 nucleotides, 1600 nucleotides, 1700 nucleotides, 1800 nucleotides, 1900 nucleotides, 2000 nucleotides, 2500 nucleotides, 3000 nucleotides, 3500 nucleotides, 4000 nucleotides, 4500 nucleotides, 5000 nucleotides, 6000 nucleotides, 7000 nucleotides, 8000 nucleotides, 9000 nucleotides, 10,000 nucleotides in length

[0203] In some aspects, the TCE are peptides or peptide fragments that are, are at least, are at least about, are less than, or are less than about 3 amino acids, 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, 8 amino acids, 9 amino acids, 10 amino acids, 11 amino acids, 12 amino acids, 13 amino acids, 14 amino acids, 15 amino acids, 16 amino acids, 17 amino acids, 18 amino acids, 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 23 amino acids, 24 amino acids, 25 amino acids, 26 amino acids, 27 amino acids, 28 amino acids, 29 amino acids, 30 amino acids, 31 amino acids, 32 amino acids, 33 amino acids, 34 amino acids, 35 amino acids, 36 amino acids, 37 amino acids, 38 amino acids, 39 amino acids, 40 amino acids, 41 amino acids, 42 amino acids, 43 amino acids, 44 amino acids, 45 amino acids, 46 amino acids, 47 amino acids, 48 amino acids, 49 amino acids, 50 amino acids, 55 amino acids, 60 amino acids, 65 amino acids, 70 amino acids, 75 amino acids, 80 amino acids, 85 amino acids, 90 amino acids, 95 amino acids, 100 amino acids, 110 amino acids, 120 amino acids, 130 amino acids, 140 amino acids, 150 amino acids, 160 amino acids, 170 amino acids, 180 amino acids, 190 amino acids, 200 amino acids, 250 amino acids, 300 amino acids, 350 amino acids, 400 amino acids, 450 amino acids, 500 amino acids, 550 amino acids, 600 amino acids, 650 amino acids, 700 amino acids, 750 amino acids, 800 amino acids, 850 amino acids, 900 amino acids, 950 amino

acids, 1000 amino acids, 1100 amino acids, 1200 amino acids, 1300 amino acids, 1400 amino acids, 1500 amino acids, 1600 amino acids, 1700 amino acids, 1800 amino acids, 1900 amino acids, 2000 amino acids, 2500 amino acids, 3000 amino acids, 3500 amino acids, 4000 amino acids, 4500 amino acids, 5000 amino acids, 6000 amino acids, 7000 amino acids, 8000 amino acids, 9000 amino acids, 10,000 amino acids in length.

[0204] Further, it will be appreciated that the term “TCE” includes sequences that comprise one, two, or multiple TCEs. For example, a TCE may refer to a recombinant string of CTL and/or HTL epitopes. In some embodiments, the NS3/4A chimeric molecules disclosed herein include epitope strings to generate a CTL response against any chosen antigen/target that contains such epitopes. Optionally, HTL epitopes which are active in individuals of different HLA types, for example HTLs from tetanus (against which most individuals will already be primed) are present in the embodiments disclosed herein. Further, in some embodiments, in addition to a TCE, it may also be useful to include B cell epitopes for stimulating B cell responses and antibody production. Optionally, multiple epitope (*e.g.* multiple TCE and/or multiple TCE and BCE) conjugates can be engineered to be linked by a linker molecule. Linkers can comprise relatively neutral amino acid sequences or amino acid mimetics, such as, *e.g.*, Ala, Gly, or other neutral linkers of nonpolar amino acids or neutral polar amino acids. In certain preferred embodiments herein the neutral linker is Ala. It will be understood that the optionally present linker need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Preferred exemplary linkers are homo-oligomers of Ala or Gly. When present, the linker will usually be at least one or two residues, more usually three to six residues. Adjuvant sequences such as nucleic acids encoding HIV TAT or fragments thereof (*e.g.*, 3, 6, 9, 12, 15, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, or 60 nucleotides in length) can be employed in some embodiments. Exemplary sequences can be found in WO05039631A1, which designates the United states and was published in English, hereby expressly incorporated by reference in its entirety. Optionally, linkers and/or adjuvant sequences flank, or are juxtaposed to TCE sequences and/or NS3/4A sequences.

[0205] The compositions and methods disclosed herein relate to antigens against which it is desired to generate an immune response. For example, the compositions and methods disclosed herein are useful, for example, in generating or enhancing the

immunogenicity of TCEs derived from agents against which CD8⁺ T cell responses have been shown to play a protective role. As such, the compositions disclosed herein are useful in diseases that include but are not limited to infection and disease caused by the viruses including, but not limited to: influenza A and B viruses (FLU-A, FLU-B), human immunodeficiency type I and II viruses (HIV-I, HIV-II), Epstein-Barr virus (EBV), human T lymphotropic (or T-cell leukemia) virus type I and type II (HTLV-I, HTLV-II), human papillomaviruses types 1 to 18 (HPV-1 to HPV-18), rubella (RV), varicella-zoster (VZV), hepatitis B (HBV), hepatitis C (HCV), adenoviruses (AV), and herpes simplex virus (HSV), cytomegalovirus (CMV), poliovirus, respiratory syncytial (RSV), rhinovirus, rabies, mumps, rotavirus or measles viruses. Further, the compositions disclosed herein are useful in diseases caused by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Plasmodium*, *Toxoplasma* and *Trypanosoma* and the like.

[0206] In a like manner, the compositions and methods described herein are applicable to tumor-associated proteins (e.g., related to melanoma, breast cancer, colon cancer and the like), which could be sources for CTL epitopes. Such tumor proteins and/or peptides, include, but are not limited to, products of the MAGE-1, -2 and -3 genes, products of the c-ErbB2 (HER-2/neu) proto-oncogene, tumor suppressor and regulatory genes which could be either mutated or overexpressed such as p53, ras, myc, and RB1. Tissue specific proteins to target CTL responses to tumors such as prostatic specific antigen (PSA) and prostatic acid phosphatase (PAP) for prostate cancer, and tyrosinase for melanoma. In addition viral related proteins associated with cell transformation into tumor cells such as EBNA-1, HPV E6 and E7 are likewise applicable. A large number of peptides from some of the above proteins have been analyzed for the presence of MHC-binding motifs and for their ability to bind with high efficiency to purified MHC molecules and are the subject of pending patent applications (U.S. patent application Ser. Nos. 08/159,339 and 08/073,205, previously incorporated herein by reference)

[0207] Other exemplary sequences that can be used in part or in whole as epitopes in the embodiments are also described herein, e.g., TCE's and BCE's.

[0208] Other exemplary HTL epitopes within a HTL peptide from tetanus toxoid 830-843 having the sequence: Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu (QYIKANSKFIGITE) [SEQ ID NO: 556], malaria circumsporozoite 382-398

(KIAKMEKASSVFNVVNS) [SEQ ID NO: 557]; malaria circumsporozoite 378-398 (DIEKKIAKMEKASSVFNVVNS) [SEQ ID NO: 558], malaria circumsporozoite 326-345 (EYLNKIQNSLSTEWSPCSVT) and ovalbumin 323-336 Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu [SEQ ID NO: 559] and the influenza epitope 307-319 Pro-Lys-Tyr-Val-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr [SEQ ID NO: 560]; *Cornebacterium diptheriae* diphtheria toxin NLFQVVHWSYNRPAYSPGYV [SEQ ID NO: 561]; *Escherichia coli* OmpF FDFGLRPSTAYTKSKAKDVVE [SEQ ID NO: 567]; *Escherichia coli* OmpF DEVFATYYFNKNMSTYVDYII [SEQ ID NO: 1379]; *Escherichia coli* OmpF NKNMSTYVDYIINQIDSKNK [SEQ ID NO: 568]. In addition, suitable T helper peptides have been identified as described in pending U.S. Patent Application Ser. No. 08/121,101, hereby expressly incorporated by reference in its entirety.

[0209] Other examples of HTL-inducing peptides are those which are specific for the antigen (virus or other organism, tumor, etc.) being targeted by the CTL. For example, several HTL-inducing peptides specific for HBV have been described, such as HBc₁₋₂₀, having the sequence: Met-Asp-Ile-Asp-Pro-Tyr-Lys-Glu-Phe-Gly-Ala-Thr-Val-Glu-Leu-Leu-Ser-Phe-Leu-Pro [SEQ ID NO: 562]; peptides from the region HBc₅₀₋₆₉, which has the sequence Pro-His-His-Tyr-Ala-Leu-Arg-Gln-Ala-Ile-Leu-Cys-Trp-Gly-Glu-Leu-Met-Tyr-Leu-Ala [SEQ ID NO: 563], and from the region of HBc₁₀₀₋₁₃₉, including HBc₁₀₀₋₁₁₉ having the sequence Leu-Leu-Trp-Phe-His-Ile-Ser-Cys-Leu-Thr-Phe-Gly-Arg-Glu-Thr-Val-Ile-Glu-Tyr-Leu [SEQ ID NO: 564] (where Ile₁₁₆ is Leu in the HBV adw subtype), HBc₁₁₇₋₁₃₁ having the sequence Glu-Tyr-Leu-Val-Ser-Phe-Gly-Val-Trp-Ile-Arg-Thr-Pro-Pro-Ala [SEQ ID NO: 565], and peptide HBc₁₂₀₋₁₃₉ having the sequence Val-Ser-Phe-Gly-Val-Trp-Ile-Arg-Thr-Pro-Pro-Ala-Tyr-Arg-Pro-Pro-Asn-Ala-Pro-Ile [SEQ ID NO: 566]. See, Ferrari et al., J. Clin. Invest. 88:214-222 (1991), and U.S. Pat. No. 4,882,145, and U.S. Pat. No. 5,143,726, hereby expressly incorporated by reference in their entireties.

[0210] The skilled artisan will also appreciate that proteins containing at least one epitope, such as a TCE, useful in the embodiments disclose herein can be identified using a variety of techniques known in the art. Illustrative methods are described in, for example, Current Protocols in Immunology, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001 John Wiley & Sons, NY, N.Y.) Ausubel et al. (2001 Current Protocols in Molecular Biology, Greene Publ. Assoc. Inc. &

John Wiley & Sons, Inc., NY, N.Y.); Sambrook et al. (1989 Molecular Cloning, Second Ed., Cold Spring Harbor Laboratory, Plainview, N.Y.); Maniatis et al. (1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, N.Y.). Illustrative methods useful in this context include intracellular cytokine staining (ICS), ELISPOT, proliferation assays, cytotoxic T cell assays including chromium release or equivalent assays, and gene expression analysis using any number of polymerase chain reaction (PCR) or RT-PCR based assays.

[0211] Epitopes of the embodiments disclosed herein may be identified using any number of techniques known in the art, such as those described by: Lamb J R, et al., (1989) Rev. Infect. Dis. March-April: Suppl 2:s443-447; Lamb J R, et al. (1987) EMBO J. 6(5):1245-1249; Lamb J R, et al., (1986) Lepr. Rev. Dec.; Suppl 2:131-137; Mehra V, et al., (1986) Proc. Natl. Acad. Sci. USA 83(18): 7013-7; Horsfall A C, et al., (1991) Immunol. Today 12(7):211-3; Rothbard J B et al., (1990) Curr Top Microbiol Immunol 155:143-52; Singh H et al., (2001) Bioinformatics 17:1236-1237; DeGroot A S, et al. Vaccine 19:4385-4395; DeLalla C, et al., (1999) J. Immunol. 163:1725-1729; Cochlovius B, et al., (2000) J. Immunol. 165:4731-4741; Consogno G, et al. (2003) Blood 101:1039-1044; Roberts C G, et al. (1996) AIDS Res. Hum. Retrovir. 12:593-610; Kwok W, et al. (2001) Trends Immunol. 22:583-588; Novak E J, et al., (2001) J. Immunol. 166:6665-6670.

[0212] An epitope that is used in some embodiments described herein may comprise a naturally occurring or naturally processed epitope as defined using any number of assays known to the skilled artisan and as described herein. Assays for identifying epitopes are known to the skilled artisan and are described, for example, in Current Protocols in Immunology, John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, and Warren Strober (Eds), John Wiley & Sons, New York. N.Y. Epitopes may be identified using intracellular cytokine staining and flow cytometric analysis such as described in Hoffmeister B., et al., (2003) Methods.; 29(3):270-281; Maecker H T, et al., (2001) J Immunol Methods 255(1-2):27-40. Similarly, proteins, peptides, overlapping peptides, or pools of peptides can be used in standard chromium release and/or proliferation assays to identify epitopes.

[0213] In those cases where antigen-specific T cell lines or clones are available, for example tumor-infiltrating lymphocytes (TIL) or virus-specific CTL, these cells can be used to screen for the presence of the relevant epitopes using target cells prepared with

specific antigens. Such targets can be prepared using random, or selected synthetic peptide libraries, which would be utilized to sensitize the target cells for lysis by the CTL. Another approach to identify the relevant epitope when T cell lines or clones are available is to use recombinant DNA methodologies. Gene, or preferably cDNA, libraries from CTL-susceptible targets are first prepared and transfected into non-susceptible target cells. This allows the identification and cloning of the gene coding the protein precursor to the peptide containing the CTL epitope. The second step in this process is to prepare truncated genes from the relevant cloned gene, in order to narrow down the region that encodes for the CTL epitope. This step is optional if the gene is not too large. The third step is to prepare synthetic peptides of approximately 10-20 amino acids of length, overlapping by 5-10 residues, which are used to sensitize targets for the CTL. When a peptide, or peptides, is shown to contain the relevant epitope, smaller peptides can be prepared to establish the peptide of minimal size that contains the epitope.

[0214] Alternatively, epitopes may be defined by direct elution of peptides bound by particular MHC molecule and direct sequencing of the peptides (see, for example, Engelhard V H, et al., *Cancer J.* 2000 May; 6 Suppl. 3:S272-80). Briefly, the eluted peptides are separated using a purification method such as HPLC, and individual fractions are tested for their capacity to sensitize targets for CTL lysis or to induce proliferation of cytokine secretion in HTL. When a fraction has been identified as containing the peptide, it is further purified and submitted to sequence analysis. The peptide sequence can also be determined using tandem mass spectrometry. A synthetic peptide is then prepared and tested with the CTL or HTL to corroborate that the correct sequence and peptide have been identified

[0215] Epitopes may also be identified using computer analysis, such as the Tsites program, which searches for peptide motifs that have the potential to elicit Th responses. (See, e.g., Rothbard and Taylor, (1988) *EMBO J.* 7:93-100; Deavin et al., (1996) *Mol. Immunol.* 33:145-155, 1996). CTL peptides with motifs appropriate for binding to murine and human class I or class II MHC may be identified according to BIMAS (Parker et al., (1994) *J. Immunol.* 152:163) and other HLA peptide binding prediction analyses. Briefly, the protein sequences for example from viral or tumor cell components are examined for the presence of MHC-binding motifs. These binding motifs which exist for each MHC allele are conserved amino acid residues, usually at positions 2 (or 3) and 9 (or 10) for MHC

class I binding peptides of 9-10 residues long. Synthetic peptides are then prepared of those sequences bearing the MHC binding motifs, and subsequently are tested for their ability to bind to MHC molecules. The MHC binding assay can be carried out either using cells which express high number of empty MHC molecules (cellular binding assay), or using purified MHC molecules. Lastly, the MHC binding peptides are then tested for their capacity to induce a CTL response in naive individuals, either in vitro using human lymphocytes, or in vivo using HLA-transgenic animals. These CTL are tested using peptide-sensitized target cells, and targets that naturally process the antigen, such as viral infected cells or tumor cells. To further confirm immunogenicity, a peptide may be tested using an HLA A2 transgenic mouse model and/or any of a variety of in vitro stimulation assays.

[0216] Epitopes that are used with embodiments described herein may also be identified using a peptide motif searching program based on algorithms developed by Rammensee, et al. (Hans-Georg Rammensee, Jutta Bachmann, Niels Nikolaus Emmerich, Oskar Alexander Bachor, Stefan Stevanovic: SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics (1999) 50: 213-219); by Parker, et. al. (supra), or using methods such as those described by Doytchinova and Flower (2002) Immunol Cell Biol. 80(3):270-9 and Blythe M J, Doytchinova I A, Flower D R. (2002), Bioinformatics 18, 434-439.

[0217] In certain embodiments, an epitope may comprise a variant of a native epitope. A "variant," as used herein, is a polypeptide (or a nucleic acid encoding such a polypeptide) that differs from a native polypeptide in one or more substitutions, deletions, additions and/or insertions, such that the immunogenicity of the polypeptide is retained (i.e., the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished relative to the native polypeptide). In other words, the ability of a variant to react with antigen-specific antisera and/or T-cell lines or clones may be enhanced or unchanged, relative to the native polypeptide, or may be diminished by less than 50%, and preferably less than 20% relative to the native polypeptide. In some embodiments, the ability of a variant to react with antigen-specific antisera and/or T-cell lines or clones may be diminished by less than 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. In one embodiment the ability of a variant to react with antigen-specific antisera and/or T-cell lines or clones

may be enhanced by at least 30%, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide with antisera and/or T-cells as described herein. In some embodiments, a variant may be identified by evaluating its ability to bind to a human, murine, or nonhuman primate MHC molecule. In preferred embodiments, a variant polypeptide has a modification such that the ability of the variant polypeptide to bind to a class I or class II MHC molecule is increased relative to that of a wild type (unmodified) polypeptide. The skilled artisan recognizes that any number of class I or class II MHC molecules can be used in the context of the embodiments described herein, for example any HLA molecule as identified and available from the IMGT/HLA database.

[0218] In more embodiments, the ability of the variant TCE to bind to an HLA molecule is increased by at least 2 fold, preferably at least 3 fold, 4 fold, or 5 fold relative to that of a native polypeptide. It has been found in some embodiments that a relatively small number of substitutions (e.g., 1 to 3) within an epitope can enhance the ability of the epitope to elicit an immune response. Suitable substitutions may generally be identified by using computer programs, as described above, and the effect can be confirmed based on the reactivity of the modified polypeptide with antisera and/or T-cells as described herein. Accordingly, within certain preferred embodiments, a variant in which 1 to 3 amino acid residues within an epitope are substituted such that the ability to react with antigen-specific antisera and/or T-cell lines or clones is statistically greater than that for the unmodified polypeptide. Such substitutions are preferably located within an MHC binding site of the polypeptide, which may be identified as described above. Preferred substitutions allow increased binding to MHC class I or class II molecules.

[0219] The CTL or HTL sequences employed in the compositions and methods described herein need not be identical to specific amino acids disclosed in aforementioned disclosures, and can be selected by a variety of techniques, for example, according to certain motifs as described above. Therefore, the epitopes may be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use. Conservative substitutions are discussed above in reference to **TABLE 1**. Usually, the portion of the sequence which is

intended to substantially mimic a CTL or HTL stimulating epitope will not differ by more than about 20% from the corresponding sequence of a native, or naturally occurring antigen, when known, except where additional amino acids may be added at either terminus for the purpose of modifying the physical or chemical properties of the peptide for, e.g., ease of linking or coupling, and the like. In those situations where regions of the peptide sequences are found to be polymorphic among antigen subtypes, it may be desirable to vary one or more particular amino acids to more effectively mimic differing CTL or HTL epitopes of different antigen strains.

[0220] In some instances, it may be desirable to combine two or more epitopes that contribute to stimulating specific CTL or HTL responses in one or more patients or histocompatibility types. The epitopes in the composition can be identical or different, and together they can provide equivalent or greater biological activity than the parent peptide(s). For example, using the methods described herein, two or more peptides may define different or overlapping CTL epitopes from a particular region, e.g., the peptide region, e.g., HBenv₃₀₉₋₃₂₈; peptide region HBenv₃₂₉₋₃₄₉, HBenv₃₄₉₋₃₆₈, or peptide region HBC₉₁₋₁₁₀, which peptides can be combined in a "cocktail" to provide enhanced immunogenicity of CTL responses, and peptides can be combined with peptides having different MHC restriction elements.

Compositions

[0221] As will be understood by those skilled in the art, the nucleic acids of the embodiments disclosed herein can be single-stranded (coding or antisense), or double-stranded, and may be a DNA (genomic, cDNA, or synthetic) or RNA molecule. Additional coding or non-coding sequences may, but need not, be present within a nucleic acid of the embodiments disclosed herein, and a nucleic acid may, but need not, be linked to other molecules and/or support materials.

[0222] Embodiments of the invention also include (a) DNA vectors that contain any of the foregoing nucleic acid sequence and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing nucleic acid sequences operatively associated with a regulatory element that directs the expression of the nucleic acid; and (c) genetically engineered host cells that contain any of the foregoing nucleic acid sequences operatively associated with a regulatory element that directs the expression of the coding

sequences in the host cell. These recombinant constructs are capable of replicating autonomously in a host cell. Alternatively, the recombinant constructs can become integrated into the chromosomal DNA of a host cell. Such recombinant polynucleotides typically comprise an HCV genomic or cDNA polynucleotide of semi-synthetic or synthetic origin by virtue of human manipulation. Therefore, recombinant nucleic acids comprising these sequences and complements thereof that are not naturally occurring are provided.

[0223] Although nucleic acids encoding NS3/4A chimeric peptides or TCE sequences or nucleic acids having sequences that complement the NS3/4A chimeric sequences or TCE sequences as they appear in nature can be employed, they will often be altered, e.g., by deletion, substitution, or insertion, and can be accompanied by sequence not present in nature. As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include, but are not limited to, the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the *lac* system, the *trp* system, the TAC system, the TRC system, the major operator and promoter regions of phage A, the control regions of *fd* coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast-mating factors.

[0224] In addition, recombinant NS3/4A chimeric peptide-encoding nucleic acid sequences and their complementary sequences can be engineered so as to modify their processing or expression. For example, and not by way of limitation, the HCV nucleic acids described herein can be combined with a promoter sequence and/or ribosome binding site, or a signal sequence can be inserted upstream of chimeric polypeptide-encoding sequences so as to permit secretion of the peptide and thereby facilitate harvesting or bioavailability. Additionally, a given NS3/4A or TCE nucleic acid can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction sites or destroy preexisting ones, or to facilitate further *in vitro* modification. (See, **Examples 1 and 2**). Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis. (Hutchinson et al., *J. Biol. Chem.*, 253:6551 (1978)). The nucleic acids encoding the chimeric NS3/4A polypeptides described above, can be manipulated using

conventional techniques in molecular biology so as to create recombinant constructs that express the NS3/4A recombinant peptides.

[0225] Further, nucleic acids encoding other proteins or domains of other proteins can be joined to nucleic acids encoding an HCV peptide so as to create a fusion protein. Nucleotides encoding fusion proteins can include, but are not limited to, a full length NS3/4A sequence (SEQ. ID. NO.: 2 or SEQ. ID. NO.: 36), mutant NS3/4A sequences (e.g., SEQ. ID. NOs.: 3-11 or 40-220) or a peptide fragment of an NS3/4A sequence fused to an unrelated protein or peptide, such as for example, polyhistidine, hemagglutinin, an enzyme, fluorescent protein, or luminescent protein, as discussed below.

[0226] It was discovered that the construct "NS3/4A-pVAX" was significantly more immunogenic *in vivo* than the construct "NS3-pVAX". Surprisingly, it was also discovered that the codon-optimized NS3/4A containing construct ("MSLF1-pVAX") was more immunogenic *in vivo* than NS3/4A pVAX. The example below describes these experiments.

EXAMPLE 3

[0227] To determine whether a humoral immune response was elicited by the NS3-pVAX and NS3/4A-pVAX vectors, the expression constructs described in *Example 1* were purified using the Qiagen DNA purification system, according to the manufacturer's instructions and the purified DNA vectors were used to immunize groups of four to ten Balb/c mice. The plasmids were injected directly into regenerating tibialis anterior (TA) muscles as previously described (Davis et al., *Human Gene Therapy* 4(6):733 (1993)). In brief, mice were injected intramuscularly with 50 μ l/TA of 0.01mM cardiotoxin (Latoxan, Rosans, France) in 0.9% sterile NaCl. Five days later, each TA muscle was injected with 50 μ l PBS containing either rNS3 or DNA.

[0228] Inbred mouse strains C57/BL6 (H-2b), Balb/C (H-2d), and CBA (H-2k) were obtained from the breeding facility at Møllegaard Denmark, Charles River Uppsala, Sweden, or B&K Sollentuna Sweden. All mice were female and were used at 4-8 weeks of age. For monitoring of humoral responses, all mice received a booster injection of 50 μ l /TA of plasmid DNA every fourth week. In addition, some mice were given recombinant NS3

(rNS3) protein, which was purified, as described herein. The mice receiving rNS3 were immunized no more than twice. All mice were bled twice a month.

[0229] Enzyme immunosorbent assays (EIAs) were used to detect the presence of murine NS3-specific antibodies. These assays were performed essentially as described (Chen et al., *Hepatology* 28(1): 219 (1998)). Briefly, rNS3 was passively adsorbed overnight at 4°C to 96-well microtiter plates (Nunc, Copenhagen, Denmark) at 1 µg/ml in 50 mM sodium carbonate buffer (pH 9.6). The plates were then blocked by incubation with dilution buffer containing PBS, 2% goat serum, and 1% bovine serum albumin for one hour at 37°C. Serial dilutions of mouse sera starting at 1:60 were then incubated on the plates for one hour. Bound murine serum antibodies were detected by an alkaline phosphatase conjugated goat anti-mouse IgG (Sigma Cell Products, Saint Louis, MO) followed by addition of the substrate pNPP (1 tablet/5ml of 1M Diethanol amine buffer with 0.5 mM MgCl₂). The reaction was stopped by addition of 1M NaOH and absorbency was read at 405 nm.

[0230] After four weeks, four out of five mice immunized with NS3/4A-pVAX had developed NS3 antibodies, whereas one out of five immunized with NS3-pVAX had developed antibodies (**FIGURE 2**). After six weeks, four out of five mice immunized with NS3/4A-pVAX had developed high levels ($>10^4$) of NS3 antibodies (mean levels 10800 ± 4830) and one had a titer of 2160. Although all mice immunized with NS3-pVAX developed NS3 antibodies, none of them developed levels as high as that produced by the NS3/4A-pVAX construct (mean levels 1800 ± 805). The antibody levels elicited by the NS3/4A fusion construct were significantly higher than those induced by NS3-pVAX at six weeks (mean ranks 7.6 v.s 3.4, $p < 0.05$, Mann-Whitney rank sum test, and $p < 0.01$, Students t-test). Thus, immunization with either NS3-pVAX or NS3/4A-pVAX resulted in the production of NS3-specific antibodies, but the NS3/4A containing construct was a more potent immunogen.

[0231] A similar experiment was conducted to compare the immunogenicity of the NS3/4A-pVAX and MSLF1-pVAX constructs. To better resemble a future vaccination schedule in humans, however, the plasmids were delivered to groups of ten mice using a gene gun. In brief, plasmid DNA was linked to gold particles according to protocols supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Prior to immunization, the injection area was shaved and the immunization was performed according to the

manufacturer's protocol. Each injection dose contained 4 µg of plasmid DNA. Immunizations were performed on weeks 0, 4, and 8.

[0232] The MSLF1 gene was found to be more immunogenic than the native NS3/4A gene since NS3-specific antibodies were significantly higher in mice immunized with the MSLF1-pVAX construct at two weeks after the second and third immunization (**TABLE 3**). These results confirmed that the codon-optimized MSLF1-pVAX was a more potent B cell immunogen than NS3/4A-pVAX.

TABLE 3

Immunogen	Week	No. of injections	Mean NS3 titre	SD	Mann-Whitney
NS3/4A	2	1	0	0	NS
MSLF1	2	1	0	0	
NS3/4A	6	2	0	0	p<0.0002
MSLF1	6	2	2484	3800	
NS3/4A	10	3	60	0	p<0.0001
MSLF1	10	3	4140	4682	

[0233] The example below provides more evidence that MSLF-1 (coNS3/4a) produces a strong humoral response.

EXAMPLE 3A

[0234] To test the intrinsic immunogenicity of the different NS3 genes groups of BALB/c (H-2^d) mice were immunized with the following vectors: wtNS3/4A (wild type NS3/4a), coNS3/4A (codon-optimized NS3/4a or MSLF-1), or wtNS3/4A-SFV (wild-type NS3/4A obtained from SFV expression). Doses of 4 µg DNA was administered using the gene gun and doses of 10⁷ SFV particles were injected subcutaneously (s.c.). The mice were boosted after four weeks. The mice immunized with the wtNS3/4A-SFV developed antibodies already after the first injection suggesting a potent immunogenicity (Figures 3A and 3B). At two weeks after the second immunization most mice immunized with the coNS3/4A or wtNS3/4A-SFV vectors had developed mean antibody levels over 10³ (Figures 3A and 3B). In contrast, none of the mice immunized with the wtNS3/4A plasmid had developed detectable NS3-specific

antibodies at six weeks (Figures 3A and 3B). Thus, both codon optimization and mRNA amplification by SFV results in an increased B cell immunogenicity of the NS3/4A gene.

[0235] To indirectly compare the T helper 1 (Th1) and Th2-skewing of the T cell response primed by wtNS3/4A, coNS3/4A, and wtNS3/4A-SFV immunizations the levels of NS3-specific IgG1 (Th2) and IgG2a (Th1) antibodies were analyzed (Figures 3A and 3B). The IgG2a/IgG1-ratio in mice immunized with rNS3 with or without adjuvant was always < 1 regardless of the murine haplotype, signaling a Th2-dominated response. In contrast, mice immunized i.m. with the wtNS3 (wild-type NS3), wtNS3/4A, or coNS3/4A containing plasmids had Th1-skewed Th-cell responses evidenced by IgG2a/IgG1 ratios of > 1 (Figure 3B). Thus, codon optimization did not change the IgG subclass distribution. When genetically immunizing BALB/c mice with NS3/4A using the gene gun the subclass ratio suggested a mixed Th1/Th2 response (Figure 3B). It should be noted that the codon optimized plasmid did not display an increased in vitro stimulatory capacity of B cells, as compared to the native plasmid, suggesting that no major immune stimulatory motifs had been lost or introduced.

[0236] Immunizations using SFV primed a Th1-, or mixed Th1/Th2-like isotype distribution. The anti-NS3 IgG2a/IgG1-ratio following wtNS3/4A-SFV immunization ranged from 2.4 to 20 between different experiments providing evidence of a Th1-like response. This is similar to the previous experience with SFV vectors where a Th1-skewed IgG subclass distribution was observed.

[0237] The example below describes experiments that were performed to determine if mutant NS3/4A peptides, which lack a proteolytic cleavage site, could elicit an immune response to NS3.

EXAMPLE 4

[0238] To test if the enhanced immunogenicity of NS3/4A could be solely attributed to the presence of NS4A, or if the NS3/4A fusion protein in addition had to be cleaved at the NS3/4A junction, another set of experiments were performed. In a first experiment, the immunogenicity of the NS3-pVAX, NS3/4A-pVAX, and mutant NS3/4A constructs were compared in Balb/c mice. Mice were immunized on week 0 as described above, and, after two weeks, all mice were bled and the presence of antibodies to NS3 at a serum dilution of 1:60 was determined (**TABLE 4**). Mice were bled again on week 4. As

shown in **TABLE 4**, all the constructs induced an immune response; the mutant constructs, for example, the NS3/4A-TGT-pVAX vector was comparable to the NS3-pVAX vector (4/10 vs. 0/10; NS, Fisher's exact test). The NS3/4A-pVAX vector, however, was more potent than the mutant constructs.

TABLE 4

Weeks from 1 st immunization	No. of antibody responders to the respective immunogen after one 100µg <i>i.m</i> immunization		
	NS3-pVAX	wild-type NS3/4A-pVAX	mutant example NS3/4A-TGT-pVAX
2	0/10	17/20	4/10
4	0/10 (<60)	20/20 (2415±3715) 55% > 10 ³ 10% > 10 ⁴	10/10 (390±639) 50% > 10 ² 10% > 10 ³

[0239] During the chronic phase of infection, HCV replicates in hepatocytes, and spreads within the liver. A major factor in combating chronic and persistent viral infections is the cell-mediated immune defense system. CD4+ and CD8+ lymphocytes infiltrate the liver during the chronic phase of HCV infection, but they are incapable of clearing the virus or preventing liver damage. In addition, persistent HCV infection is associated with the onset of hepatocellular carcinoma (HCC). The examples below describe experiments that were performed to determine whether the NS3, NS3/4A, and MSLF1 constructs were capable of eliciting a T-cell mediated immune response against NS3.

EXAMPLE 5

[0240] To study whether the constructs described above were capable of eliciting a cell-mediated response against NS3, an *in vivo* tumor growth assay was performed. To this end, an SP2/0 tumor cell line (SP2/0-Ag14 myeloma cell line (H-2^d)) stably transfected with the NS3/4A gene was made. The SP2/0 cells were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS; Sigma Chemicals, St. Louis, MO), 2 mM L-Glutamine, 10mM HEPES, 100 U/ml Penicillin and 100 µg/ml Streptomycin, 1mM non-

essential amino acids, 50 μ M β -mercaptoethanol, 1mM sodium pyruvate (GIBCO-BRL, Gaithersburgh, MD). The pcDNA3.1 plasmid containing the NS3/4A gene was linearized by *Bgl*III digestion. A total of 5 μ g linearized plasmid DNA was mixed with 60 μ g transfection reagent (Superfect, Qiagen, Germany) and the mixture was added to a 50% confluent layer of SP2/0 cells in a 35 mm dish. The transfection procedure was performed according to manufacturer's protocol.

[0241] Transfected cells were cloned by limiting dilution and selected by addition of 800 μ g geneticin (G418) /ml complete DMEM medium after 14 days. A stable NS3/4A-expressing SP2/0 clone was identified using PCR and RTPCR and/or a capture EIA using a monoclonal antibody to NS3. All EIAs for the detection of murine NS3 antibodies were essentially performed as follows. In brief, rNS3 (recombinant NS3 protein produced in *E. Coli*, dialyzed overnight against PBS, and sterile filtered) was passively adsorbed overnight at 4°C to 96-well microtiter plates (Nunc, Copenhagen, Denmark) at 1 μ g/ml in 50 mM sodium carbonate buffer (pH 9.6). The plates were then blocked by incubation with dilution buffer containing PBS, 2% goat serum, and 1% bovine serum albumin for one hour at +37°C. Serial dilutions of mouse sera starting at 1:60 were then incubated on the plates for one hour. Bound murine serum antibodies were detected by an alkaline phosphatase conjugated goat anti-mouse IgG (Sigma cellproducts, Saint Louis, Missouri USA) followed by addition of the substrate pNPP (1 tablet/5ml of 1M Diethanolamine buffer with 0.5 mM MgCl₂). The reaction was stopped by addition of 1M NaOH. Absorbance was then read at 405 nm.

[0242] The *in vivo* growth kinetics of the SP2/0 and the NS3/4A-SP2/0 cell lines were then evaluated in Balb/c mice. Mice were injected subcutaneously with 2 x 10⁶ tumor cells in the right flank. Each day the size of the tumor was determined through the skin. The growth kinetics of the two cell lines was comparable. The mean tumor sizes did not differ between the two cell lines at any time point, for example. (See **TABLE 5**).

TABLE 5

Mouse ID	Tumor cell line	Maximum in vivo tumor size at indicated time point								
		5	6	7	8	11	12	13	14	15
1	SP2/0	1.6	2.5	4.5	6.0	10.0	10.5	11.0	12.0	12.0
2	SP2/0	1.0	1.0	2.0	3.0	7.5	7.5	8.0	11.5	11.5
3	SP2/0	2.0	5.0	7.5	8.0	11.0	11.5	12.0	12.0	13.0
4	SP2/0	4.0	7.0	8.0	10.0	13.0	15.0	16.5	16.5	17.0
5	SP2/0	1.0	1.0	3.0	4.0	5.0	6.0	6.0	6.0	7.0
Group mean		1.92	3.3	5.0	6.2	9.3	10.1	10.7	11.6	12.1
6	NS3/4 A-SP2/0	1.0	2.0	3.0	3.5	4.0	5.5	6.0	7.0	8.0
7	NS3/4 A-SP2/0	2.0	2.5	3.0	5.0	7.0	9.0	9.5	9.5	11.0
8	NS3/4 A-SP2/0	1.0	2.0	3.5	3.5	9.5	11.0	12.0	14.0	14.0
9	NS3/4 A-SP2/0	1.0	1.0	2.0	6.0	11.5	13.0	14.5	16.0	18.0
10	NS3/4 A-SP2/0	3.5	6.0	7.0	10.5	15.0	15.0	15.0	15.5	20.0
Group mean		1.7	2.7	3.7	5.7	9.4	10.7	11.4	12.4	14.2
p-value of student's t-test comparison between group means		0.7736	0.6918	0.402 ₇	0.7903	0.9670	0.798 ₆	0.7927	0.7508	0.462 ₃

[0243] The example below describes experiments that were performed to determine whether mice immunized with the NS3/4A constructs had developed a T-cell response against NS3.

EXAMPLE 6

[0244] To examine whether a T-cell response was elicited by the NS3/4A immunization, the capacity of an immunized mouse's immune defense system to attack the NS3-expressing tumor cell line was assayed. The protocol for testing for *in vivo* inhibition of tumor growth of the SP2/0 myeloma cell line in Balb/c mice has been described in detail

previously (Encke et al., *J. Immunol.* 161:4917 (1998)). Inhibition of tumor growth in this model is dependent on the priming of cytotoxic T lymphocytes (CTLs). In a first set of experiments, groups of ten mice were immunized *i.m.* five times with one month intervals with either 100µg NS3-pVAX or 100 µg NS3/4A-pVAX. Two weeks after the last immunization 2×10^6 SP2/0 or NS3/4A-SP2/0 cells were injected into the right flank of each mouse. Two weeks later the mice were sacrificed and the maximum tumor sizes were measured. There was no difference between the mean SP2/0 and NS3/4A-SP2/0 tumor sizes in the NS3-pVAX immunized mice. (See **TABLE 6**).

TABLE 6

Mouse ID	Immunogen	Dose (µg)	Tumor cell line	Tumor growth	Maximum tumor size (mm)
1	NS3-pVAX	100	SP2/0	Yes	5
2	NS3-pVAX	100	SP2/0	Yes	15
3	NS3-pVAX	100	SP2/0	No	-
4	NS3-pVAX	100	SP2/0	Yes	6
5	NS3-pVAX	100	SP2/0	Yes	13
Group total				4/5	9.75±4.992
6	NS3-pVAX	100	NS3/4A-SP2/0	Yes	9
7	NS3-pVAX	100	NS3/4A-SP2/0	Yes	8
8	NS3-pVAX	100	NS3/4A-SP2/0	Yes	7
9	NS3-pVAX	100	NS3/4A-SP2/0	No	-
10	NS3-pVAX	100	NS3/4A-SP2/0	No	-
				3/5	8.00±1.00

Note: Statistical analysis (StatView): Student's t-test on maximum tumor size. P-values < 0.05 are considered significant.

Unpaired t-test for Max diam

Grouping Variable: Column 1

Hypothesized Difference = 0

Row exclusion: NS3DNA-Tumor-001213

	Mean Diff.	DF	t-Value	P-Value
NS3-sp2, NS3-spNS3	1.750	5	0.58	0.584

Group Info for Max diam**Grouping Variable: Column 1****Row exclusion: NS3DNA-Tumor-001213**

	Count	Mean	Variance	Std. Dev.	Std. Err
NS3-sp2	4	9.750	24.917	4.992	2.496
NS3-spNS3	3	8.000	1.000	1.000	0.57

[0245] To analyze whether administration of different NS3 containing compositions affected the elicitation of a cell-mediated immune response, mice were immunized with PBS, rNS3, a control DNA, or the NS3/4A construct, and tumor sizes were determined, as described above. The NS3/4A construct was able to elicit a T-cell response sufficient to cause a statistically significant reduction in tumor size (*See TABLE 7*).

TABLE 7

Mouse ID	Immunogen	Dose (μg)	Tumor cell line	Anti-NS3	Tumor growth	Maximum tumor size (mm)
1	NS3-pVAX	10	NS3/4A-SP2/0	<60	+	12.0
2	NS3-pVAX	10	NS3/4A-SP2/0	<60	+	20.0
3	NS3-pVAX	10	NS3/4A-SP2/0	60	+	18.0
4	NS3-pVAX	10	NS3/4A-SP2/0	<60	+	13.0
5	NS3-pVAX	10	NS3/4A-SP2/0	<60	+	17.0
Group mean				60	5/5	16.0±3.391
6	NS3-pVAX	100	NS3/4A-SP2/0	2160	+	10.0
7	NS3-pVAX	100	NS3/4A-SP2/0	<60	-	-
8	NS3-pVAX	100	NS3/4A-SP2/0	<60	-	-
9	NS3-pVAX	100	NS3/4A-SP2/0	360	-	-
10	NS3-pVAX	100	NS3/4A-SP2/0	<60	+	12.5
Group mean				1260	2/5	11.25±1.768
11	NS3/4A-pVAX	10	NS3/4A-SP2/0	<60	+	10.0
12	NS3/4A-pVAX	10	NS3/4A-SP2/0	<60	-	-
13	NS3/4A-pVAX	10	NS3/4A-SP2/0	<60	-	-
14	NS3/4A-pVAX	10	NS3/4A-SP2/0	<60	+	13.0
15	NS3/4A-pVAX	10	NS3/4A-SP2/0	<60	+	13.5
Group mean				<60	3/5	12.167±1.893
16	NS3/4A-pVAX	100	NS3/4A-SP2/0	60	+	10.0
17	NS3/4A-pVAX	100	NS3/4A-SP2/0	360	-	-
18	NS3/4A-pVAX	100	NS3/4A-SP2/0	2160	+	8.0
19	NS3/4A-pVAX	100	NS3/4A-SP2/0	2160	+	12.0
20	NS3/4A-pVAX	100	NS3/4A-SP2/0	2160	+	7.0
Group mean				1380	4/5	9.25±2.217
36	p17-pcDNA3	100	NS3/4A-SP2/0	<60	+	20.0
37	p17-pcDNA3	100	NS3/4A-SP2/0	<60	+	7.0
38	p17-pcDNA3	100	NS3/4A-SP2/0	<60	+	11.0
39	p17-pcDNA3	100	NS3/4A-SP2/0	<60	+	15.0
40	p17-pcDNA3	100	NS3/4A-SP2/0	<60	+	18.0
Group mean				<60	5/5	14.20±5.263
41	rNS3/CFA	20	NS3/4A-SP2/0	>466560	+	13.0
42	rNS3/CFA	20	NS3/4A-SP2/0	>466560	-	-
43	rNS3/CFA	20	NS3/4A-SP2/0	>466560	+	3.5
44	rNS3/CFA	20	NS3/4A-SP2/0	>466560	+	22.0
45	rNS3/CFA	20	NS3/4A-SP2/0	>466560	+	17.0
Group mean				466560	4/5	17.333±4.509
46	PBS	-	NS3/4A-SP2/0	<60	+	10.0
47	PBS	-	NS3/4A-SP2/0	<60	+	16.5

Mouse ID	Immunogen	Dose (µg)	Tumor cell line	Anti-NS3	Tumor growth	Maximum tumor size (mm)
48	PBS	-	NS3/4A-SP2/0	60	+	15.0
49	PBS	-	NS3/4A-SP2/0	<60	+	21.0
50	PBS	-	NS3/4A-SP2/0	<60	+	15.0
51	PBS	-	NS3/4A-SP2/0	<60	-	-
Group mean				60	5/6	15.50±3.937

Note: Statistical analysis (StatView): Student's t-test on maximum tumor size. P-values < 0.05 are considered as significant.

Unpaired t-test for Largest Tumor size

Grouping Variable: group

Hypothesized Difference = 0

	Mean Diff.	DF	t-Value	P-Value
p17-sp3-4, NS3-100-sp3-4	2.950	5	.739	.4933
p17-sp3-4, NS3/4-10-sp3-4	2.033	6	.628	.5532
p17-sp3-4, NS3-10-sp3-4	-1.800	8	-.643	.5383
p17-sp3-4, NS3/4-100-sp3-4	4.950	7	1.742	.1250
p17-sp3-4, PBS-sp3-4	-1.300	8	-.442	.6700
p17-sp3-4, rNS3-sp3-4	-3.133	6	-.854	.4259
NS3-100-sp3-4, NS3/4-10-sp3-4	-.917	3	-.542	.6254
NS3-100-sp3-4, NS3-10-sp3-4	-4.750	5	-1.811	.1299
NS3-100-sp3-4, NS3/4-100-sp3-4	2.000	4	1.092	.3360
NS3-100-sp3-4, PBS-sp3-4	-4.250	5	-1.408	.2183
NS3-100-sp3-4, rNS3-sp3-4	-6.083	3	-1.744	.1795
NS3/4-10-sp3-4, NS3-10-sp3-4	-3.833	6	-1.763	.1283
NS3/4-10-sp3-4, NS3/4-100-sp3-4	2.917	5	1.824	.1277
NS3/4-10-sp3-4, PBS-sp3-4	-3.333	6	-1.344	.2274
NS3/4-10-sp3-4, rNS3-sp3-4	-5.167	4	-1.830	.1412
NS3-10-sp3-4, NS3/4-100-sp3-4	6.750	7	3.416	.0112
NS3-10-sp3-4, PBS-sp3-4	.500	8	.215	.8350
NS3-10-sp3-4, rNS3-sp3-4	-1.333	6	-.480	.6480
NS3/4-100-sp3-4, PBS-sp3-4	-6.250	7	-2.814	.0260
NS3/4-100-sp3-4, rNS3-sp3-4	-8.083	5	-3.179	.0246
PBS-sp3-4, rNS3-sp3-4	-1.833	6	-.607	.5662

[0246] The example below describes more experiments that were performed to determine whether the reduction in tumor size can be attributed to the generation of NS3-specific T-lymphocytes.

EXAMPLE 7

[0247] In the next set of experiments, the inhibition of SP2/0 or NS3/4A-SP2/0 tumor growth was again evaluated in NS3/4A-pVAX immunized Balb/c mice. In mice immunized with the NS3/4A-pVAX plasmid, the growth of NS3/4A-SP2/0 tumor cells was significantly inhibited as compared to growth of the non-transfected SP2/0 cells. (See **TABLE 8**). Thus, NS3/4A-pVAX immunization elicits CTLs that inhibit growth of cells expressing NS3/4A *in vivo*.

TABLE 8

Mouse ID	Immunogen	Dose (µg)	Tumor cell line	Tumor growth	Maximum tumor size (mm)
11	NS3/4A-pVAX	100	SP2/0	No	-
12	NS3/4A-pVAX	100	SP2/0	Yes	24
13	NS3/4A-pVAX	100	SP2/0	Yes	9
14	NS3/4A-pVAX	100	SP2/0	Yes	11
15	NS3/4A-pVAX	100	SP2/0	Yes	25
				4/5	17.25±8.421
16	NS3/4A-pVAX	100	NS3/4A-SP2/0	No	-
17	NS3/4A-pVAX	100	NS3/4A-SP2/0	Yes	9
18	NS3/4A-pVAX	100	NS3/4A-SP2/0	Yes	7
19	NS3/4A-pVAX	100	NS3/4A-SP2/0	Yes	5
20	NS3/4A-pVAX	100	NS3/4A-SP2/0	Yes	4
				4/5	6.25±2.217

Note: Statistical analysis (StatView): Student's t-test on maximum tumor size. P-values

< 0.05 are considered significant.

Unpaired t-test for Max diam

Grouping Variable: Column 1

Hypothesized Difference = 0

Row exclusion: NS3DNA-Tumor-001213

	Mean Diff.	DF	t-Value	P-Value
NS3/4-sp2, NS3/4-spNS3	11.000	6	2.526	0.044

Group Info for Max diam

Grouping Variable: Column 1

Row exclusion: NS3DNA-Tumor-001213

	Count	Mean	Variance	Std. Dev.	Std. Err
NS3/4-sp2	4	17.250	70.917	8.421	4.211
NS3/4-spNS3	4	6.250	4.917	2.217	1.109

[0248] In another set of experiments, the inhibition of NS3/4A-expressing SP2/0 tumor growth was evaluated in MSLF1-pVAX immunized Balb/c mice. In brief, groups of mice were immunized with different immunogens (4 μ g of plasmid) using a gene gun at weeks zero, four, eight, twelve, and sixteen. Two weeks after the last immunization approximately 2×10^6 NS3/4A-expressing SP2/0 cells were injected *s.c* into the right flank of the mouse. The kinetics of the tumor growth was then monitored by measuring the tumor size through the skin at days seven, 11, and 13. The mean tumor sizes were calculated and groups were compared using the Mann-Whitney non-parametric test. At day 14 all mice were sacrificed.

[0249] After only a single immunization, tumor inhibiting responses were observed. (See **FIGURE 3** and **TABLE 9**). After two immunizations, both the NS3/4A-pVAX and MSLF1-pVAX plasmids primed tumor-inhibiting responses. (See **FIGURE 4A** and **TABLE 10**). The tumors were significantly smaller in mice immunized with the MSLF1 gene, however, as compared to the native NS3/4A gene. After three injections, both plasmids effectively primed comparable tumor inhibiting responses. (See **FIGURE 4B** and **TABLE 11**). These experiments provided evidence that the MSLF-1 gene was more efficient in activating tumor inhibiting immune responses *in vivo* than NS3/4A-pVAX.

TABLE 9

Group	MSLF1-pVAX1	NS3/4A-pVAX1	Non-immunized
MSLF1-pVAX1	-	N.S.	p<0,05
NS3/4A-pVAX1	N.S.	-	p<0,05
Non-immunized	p<0,05	p<0,05	-

TABLE 10

Group	MSLF1-pVAX1	NS3/4A-pVAX1	Non-immunized
MSLF1-pVAX1	-	p<0,05	p<0,01
NS3/4A-pVAX1	p<0,05	-	p<0,01
Non-immunized	p<0,01	p<0,01	-

TABLE 11

Group	MSLF1-pVAX1	NS3/4A-pVAX1	Non-immunized
MSLF1-pVAX1	-	N.S.	p<0,01
NS3/4A-pVAX1	N.S.	-	p<0,01
Non-immunized	p<0,01	p<0,01	-

[0250] The example below describes experiments that were performed to analyze the efficiency of various NS3 containing compositions in eliciting a cell-mediated response to NS3.

EXAMPLE 8

[0251] To determine whether NS3-specific T-cells were elicited by the NS3/4A immunizations, an *in vitro* T-cell mediated tumor cell lysis assay was employed. The assay has been described in detail previously (Sallberg et al., *J. Virol.* 71:5295 (1997)). In a first set of experiments, groups of five Balb/c mice were immunized three times with 100µg NS3/4A-pVAX *i.m.* Two weeks after the last injection the mice were sacrificed and splenocytes were harvested. Re-stimulation cultures with 3×10^6 splenocytes and 3×10^6 NS3/4A-SP2/0 cells were set. After five days, a standard Cr⁵¹-release assay was performed using NS3/4A-SP2/0 or SP2/0 cells as targets. Percent specific lysis was calculated as the ratio between lysis of NS3/4A-SP2/0 cells and lysis of SP2/0 cells. Mice immunized with NS3/4A-pVAX displayed specific lysis over 10% in four out of five tested mice, using an effector to target ratio of 20:1 (See **FIGURES 6A** and **6B**).

[0252] In a next set of experiments, the T cell responses to MSLF1-pVAX and NS3/4A-pVAX were compared. The ability of the two plasmids to prime *in vitro* detectable

CTLs were evaluated in C57/BL6 mice since an H-2b-restricted NS3 epitope had been previously mapped. Groups of mice were immunized with the two plasmids and CTLs were detected *in vitro* using either peptide coated H-2b expressing RMA-S cells or NS3/4A-expressing EL-4 cells. Briefly, *in vitro* stimulation was carried out for five days in 25-ml flasks at a final volume of 12 ml, containing 5U/ml recombinant murine IL-2 (mIL-2; R&D Systems, Minneapolis, MN). The restimulation culture contained a total of 40×10^6 immune spleen cells and 2×10^6 irradiated (10,000 rad) syngenic SP2/0 cells expressing the NS3/4A protein. After five days *in vitro* stimulation a standard ^{51}Cr -release assay was performed. Effector cells were harvested and a four-hour ^{51}Cr assay was performed in 96-well U-bottom plates in a total volume of 200 μl . A total of 1×10^6 target cells was labeled for one hour with 20 μl of ^{51}Cr (5 mCi/ml) and then washed three times in PBS. Cytotoxic activity was determined at effector:target (E:T) ratios of 40:1, 20:1, and 10:1, using 5×10^3 ^{51}Cr -labeled target cells/well.

[0253] Alternatively, spleenocytes were harvested from C57BL/6 mice 12 days after peptide immunization and were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-Glutamine, 10mM HEPES, 100 U/ml Penicillin and 100 $\mu\text{g}/\text{ml}$ Streptomycin, 1mM non-essential amino acids, 50 μM β -mercaptoethanol, 1mM sodium pyruvate. *In vitro* stimulation was carried out for five days in 25ml flasks in a total volume of 12ml, containing 25×10^6 spleen cells and 25×10^6 irradiated (2,000 rad) syngeneic splenocytes. The restimulation was performed in the presence of 0.05 μM NS3/4A H-2D^b binding peptide (sequence GAVQNEVTI **SEQ. ID. NO.: 37**) or a control peptide H-2D^b peptide (sequence KAVYNFATM **SEQ. ID. NO.: 38**). After five days a ^{51}Cr -release assay was performed. RMA-S target cells were pulsed with 50 μM peptide for 1.5 hrs at +37°C prior to ^{51}Cr -labelling, and then washed three times in PBS. Effector cells were harvested and the four hour ^{51}Cr assay was performed as described. Cytotoxic activity was determined at the E:T ratios 60:1, 20:1, and 7:1 with 5×10^3 ^{51}Cr -labeled target cells/well. By these assays, it was determined that the MSLF1 gene primed higher levels of *in vitro* lytic activity compared to the NS3/4A-pVAX vector. (See **FIGURE 7A-7L**). Similar results were obtained with both the peptide coated H-2b expressing RMA-S cells and NS3/4A-expressing EL-4 cells.

[0254] Additional evidence that the codon-optimized MSLF1 gene primed NS3-specific CTLs more effectively than the native NS3/4A gene was obtained using flow cytometry. The frequency of NS3/4A-peptide specific CD8⁺ T cells were analyzed by *ex-vivo* staining of spleen cells from NS3/4A DNA immunized mice with recombinant soluble dimeric mouse H-2D^b:Ig fusion protein. Many of the monoclonal antibodies and MHC:Ig fusion proteins described herein were purchased from BDB Pharmingen (San Diego, CA); Anti-CD16/CD32 (Fc-block™, clone 2.4G2), FITC conjugated anti-CD8 (clone 53-6.7), FITC conjugated anti-H-2K^b (clone AF6-88.5), FITC conjugated anti-H-2D^b (clone KH95), recombinant soluble dimeric mouse H-2D^b:Ig, PE conjugated Rat-α Mouse IgG1 (clone X56).

[0255] Approximately, 2×10^6 spleen cells resuspended in 100 μl PBS/1% FCS (FACS buffer) were incubated with 1 μg/ 10^6 cells of Fc-blocking antibodies on ice for 15 minutes. The cells were then incubated on ice for 1.5 hrs with either 2 μg/ 10^6 cells of H-2D^b:Ig preloaded for 48 hours at +4°C with 640 nM excess of NS3/4A derived peptide (sequence GAVQNEVTI **SEQ. ID. NO.: 37**) or 2 μg/ 10^6 cells of unloaded H-2D^b:Ig fusion protein. The cells were then washed twice in FACS buffer and resuspended in 100 μl FACS buffer containing 10 μl/100μl PE conjugated Rat-α Mouse IgG1 secondary antibody and incubated on ice for 30 minutes. The cells were then washed twice in FACS buffer and incubated with 1μg/ 10^6 cells of FITC conjugated α-mouse CD8 antibody for 30 minutes. The cells were then washed twice in FACS buffer and resuspended in 0.5 ml FACS buffer containing 0.5 μg/ml of PI. Approximately 200,000 events from each sample were acquired on a FACS Calibur (BDB) and dead cells (PI positive cells) were excluded from the analysis.

[0256] The advantage of quantifying specific CTLs by FACS analysis is that it bypasses the possible disadvantages of *in vitro* expansion of CTLs in vitro prior to analysis. Direct *ex-vivo* quantification of NS3-specific CTLs using NS3-peptide loaded divalent H-2D^b:Ig fusion protein molecules revealed that the codon optimized MSLF-1 gene primed a effectively primed NS3-specific CTLs already after two immunizations, whereas the original NS3/4A gene did not. Thus, the optimized MSLF-1 gene effectively primes NS3-specific CTLs that are of higher frequency and of better functionality by all parameters tested, as

compared to the original NS3/4A gene. The example below provides more evidence that codon optimized NS3/4A efficiently primes NS3 specific cytotoxic T cells.

EXAMPLE 8A

[0257] Initially, the frequency of NS3-specific CTLs that could be primed by gene gun immunization using the wtNS3, wtNS3/4A and coNS3/4A expressing plasmids was determined. The coNS3/4A plasmid primed higher precursor frequencies of NS3-specific CTL as compared to the wtNS3 gene enforcing the importance of NS4A (**FIGURE 8A**). No statistical difference in CTL precursor frequencies was noted between the wtNS3/4A and coNS3/4A expressing plasmids when analyzed directly *ex vivo* (**FIGURE 8A**). A single immunization with the coNS3/4A plasmid or wtNS3/4A-SFV primed around 1% of peptide-specific CTLs within two weeks from immunization (**FIGURE 8A**). The specificity of the detection of NS3-specific CTLs was confirmed by a five-day restimulation *in vitro* with the NS3-peptide, by which high precursor frequencies were observed after immunization with the coNS3/4A gene (**FIGURE 8A**).

[0258] To directly compare the *in vitro* lytic activity of the NS3-specific CTLs primed by different vectors, a standard ^{51}Cr -release assay was performed after one or two immunizations. The lytic activity of the *in vivo* primed CTLs were assayed on both NS3-peptide loaded H-2D^b expressing RMA-S cells and EL-4 cells stably expressing NS3/4A. After one dose, the coNS3/4A plasmid and the wtNS3/4A-SFV vector was clearly more efficient than the wtNS3/4A plasmid in priming CTLs that lysed NS3-peptide coated target cells (FIGURES 9A and 9B). Thus, the CTL priming event was enhanced by codon optimization or mRNA amplification of the NS3/4A gene. The difference was less clear when using the NS3/4A-expressing EL-4 cells presumably since this assay is less sensitive (FIGURES 9A and 9B). After two immunizations all NS3/4A vectors seemed to prime NS3-specific CTLs with a similar efficiency (FIGURE 9B). However, two immunizations with any of the NS3/4A-containing vectors were clearly more efficient in priming NS3-specific CTLs as compared to the plasmid containing only the wtNS3 gene (FIGURE 9B), which is fully consistent with the CTL precursor analysis and previous observations. Thus, codon optimization or mRNA amplification of the NS3/4A gene more rapidly primes NS3-specific CTLs.

[0259] Analysis of the inhibition of tumor growth in vivo in BALB/c mice using SP2/0 myeloma cells, or in C57BL/6 mice using EL-4 lymphoma cells, expressing an HCV viral antigen is recognized by those in the field to represent the in vivo functional HCV-specific immune response. (See Encke J et al., J Immunol 161: 4917-4923 (1998)). An SP2/0 cell line stably expressing NS3/4A has previously been described (see Frelin L et al., Gene Ther 10: 686-699 (2003)) and an NS3/4A expressing EL-4 cell line was characterized as described below.

[0260] To confirm that inhibition of tumor growth using the NS3/4A-expressing EL-4 cell line is fully dependent on an NS3/4A-specific immune response a control experiment was performed. Groups of ten C57BL/6 mice were either left nonimmunized, or immunized twice with the coNS3/4A plasmid. Two weeks after the last immunization the mice were challenged with an s.c. injection of 10^6 native EL-4 or NS3/4A-expressing EL-4 cells (NS3/4A-EL-4). An NS3/4A-specific immune response was required for protection, since only the immunized mice were protected against growth of the NS3/4A-EL-4 cell line (FIGURE 10A). Thus, this H-2^b-restricted model behaves similarly to the SP2/0 H-2^d restricted model.

[0261] Immunizations with recombinant NS3 protein provided evidence that both NS3/4A-specific B cells and CD4⁺ T cells were not of a pivotal importance in protection against tumor growth. In vitro depletion of CD4⁺ or CD8⁺ T cells of splenocytes from coNS3/4A plasmid immunized H-2^b mice provided evidence that CD8⁺ T cells were the major effector cells in the ⁵¹Cr-release assay. To define the in vivo functional anti-tumor effector cell population, CD4⁺ or CD8⁺ T cells in mice immunized with the coNS3/4A plasmid one week prior to, and during, challenge with the NS3/4A-EL-4 tumor cell line were selectively depleted. Analysis by flow cytometry revealed that 85% of CD4⁺ and CD8⁺ T cells had been depleted, respectively. This experiment revealed that in vivo depletion of CD4⁺ T cells had no significant effect on the tumor immunity (FIGURE 10B). In contrast, depletion of CD8⁺ T cells in vivo significantly reduced the tumor immunity ($p < 0.05$, ANOVA; FIGURE 10B). Thus, as expected, NS3/4A-specific CD8⁺ CTLs seems to be the major protective cell at the effector stage in the in vivo model for inhibition of tumor growth.

[0262] The tumor challenge model was then used to evaluate how effective the different immunogens were in priming a protective immunity against growth of NS3/4A-EL-4 tumor cells in vivo. To ensure that the effectiveness of the priming event was studied, all mice were immunized only once. Fully consistent with the in vitro CTL data did we find that only

vectors containing NS3/4A were able to rapidly prime protective immune responses as compared to the immunized with the empty pVAX plasmid ($p < 0.05$, ANOVA; FIGURE 11). However, this was dependent on NS4A but independent of either codon optimization or mRNA amplification, suggesting that C57BL/6 mice are quite easily protected against tumor growth using genetic immunization.

[0263] To further clarify the prerequisites for priming of the in vivo protective CD8⁺ CTL responses additional experiments were performed. First, C57BL/6 mice immunized with the NS3-derived CTL peptide were not protected against growth of NS3/4A-EL-4 tumors (FIGURE 11). Second, immunization with recombinant NS3 in adjuvant did not protect against tumor growth (FIGURE 11). NS3-derived CTL peptide effectively primes CTLs in C57BL/6 mice and rNS3 in adjuvant primes high levels of NS3-specific T helper cells. Thus, an endogenous production of NS3/4A seems to be needed to prime in vivo protective CTLs. To further characterize the priming event, groups of B cell (μ MT) or CD4 deficient C57BL/6 mice were immunized once with the coNS3/4A gene using gene gun, and were challenged two weeks later (FIGURE 11). Since both lineages were protected against tumor growth we conclude that neither B cells nor CD4⁺ T cells were required for the priming of in vivo functional NS3/4A-specific CTLs (FIGURE 11). In conclusion, the priming of in vivo tumor protective NS3/4A-specific CTLs in C57BL/6 mice requires NS4A and an endogenous expression of the immunogen. In C57BL/6 mice the priming is less dependent on the gene delivery route or accessory cells, such as B cells or CD4⁺ T cells. The fact that the priming of in vivo functional CTL by the coNS3/4A DNA plasmid was independent of CD4⁺ T helper cells may help to explain the speed by which the priming occurred.

[0264] Repeated experiments in C57BL/6 mice using the NS3/4A-EL-4 cell line have shown that protection against tumor growth is obtained already after the first immunization with the NS3/4A gene, independent of codon optimization or mRNA amplification. Also, after two injections the immunity against NS3/4A-EL-4 tumor growth was even further enhanced, but only when NS4A was present. Thus, this model may therefore not be sufficiently sensitive to reveal subtle differences in the intrinsic immunogenicity of different immunogens.

[0265] To better compare the immunogenicity of the wtNS3/4A and the coNS3/4A DNA plasmids, additional experiments were performed in H-2^d mice, where at least two immunizations seemed to be required for a tumor protective immunity. It is important to

remember that the IgG subclass distribution obtained after gene gun immunization with the NS3/4A gene in BALB/c mice suggested a mixed Th1/Th2-like response. Thus, it was possible that a Th2-like immunization route (gene gun) in the Th2-prone BALB/c mouse strain may impair the ability to prime in vivo effective CTL responses.

[0266] Groups of ten BALB/c mice were immunized once, twice, or thrice with 4 μ g of the respective DNA plasmid using the gene gun (FIGURES 12A – 12C). The mice were challenged two weeks after the last injection. Accordingly, these experiments provided more evidence that the coNS3/4A plasmid primed an in vivo functional NS3/4A-specific tumor inhibiting immunity more rapidly than the wild type plasmid (FIGURES 12A – 12C). Two doses of the coNS3/4A primed a significantly better NS3/4A-specific tumor inhibiting immunity as compared to the wtNS3/4A plasmid ($p < 0.05$, ANOVA; FIGURES 12A – 12C). After three doses the tumor inhibiting immunity was the same. Thus, the data above verified that the codon optimization of the NS3/4A gene primes NS3-specific CTLs more rapidly.

[0267] As set forth herein, the NS3/4A gene can be used as a vaccine. Although it had been determined that NS3/4A quickly primed in vivo functional CTLs, the effect of therapeutic immunization after the injection of tumor cells was analyzed next. Groups of ten C57BL/6 mice were challenged with 10^6 NS3/4A-EL-4 tumor cells. One group was immunized transdermally with 4 μ g coNS3/4A at six days, and another group at 12 days, after tumor challenge. After the therapeutic vaccination both groups had significantly smaller tumors as compared to the nonimmunized control group ($p < 0.01$, respectively, ANOVA; FIGURE 13). This confirms that the vaccine rapidly primes CTLs, which are able to home to and infiltrate the NS3/4A-expressing tumors. Thus, gene gun immunization with the coNS3/4A plasmid also works as a therapeutic vaccine. That is, gene gun immunization using the coNS3/4A gene six to 12 days after inoculation of NS3/4A-expressing tumor cells significantly inhibited tumor growth. Overall, a rapid priming of HCV NS3-specific immune responses that are functional in vivo are generated by either DNA based immunization with a codon optimized gene or by mRNA amplification by the SFV replicon. By using these approaches, one can prepare very effective vaccines for the treatment and prevention of chronic HCV infections. The next example described in greater detail some of the materials and methods used in the experiments described herein.

EXAMPLE 8B

Mice

[0268] Inbred BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from commercial vendors (Møllegaard, Denmark). B cell (μ MT) deficient mice were kindly provided by Dr Karin Sandstedt, Karolinska Institutet, Sweden. CD4 deficient C57BL/6 mice were obtained from the breeding facility at the Microbiology and Tumorbiology Centre, Karolinska Institutet. All mice were female and were used at 4-8 weeks of age at the start of the experiments.

Recombinant NS3 ATPase/helicase domain protein

[0269] The recombinant NS3 (rNS3) protein was kindly provided by Darrell L. Peterson, Department of Biochemistry, Commonwealth University, VA. The production of recombinant NS3 protein (not including NS4A) in E. Coli has been described in the field. Prior to use the rNS3 protein was dialyzed over night against PBS and sterile filtered.

Generation of a synthetic codon optimized (co) NS3/4A gene

[0270] The sequence of the previously isolated and sequenced unique wtNS3/4A gene was analyzed for codon usage with respect to the most commonly used codons in human cells. A total of 435 nucleotides were replaced to optimize codon usage for human cells. The sequence was sent to Retrogen Inc (San Diego, CA) for generation of a full-length synthetic coNS3/4A gene. The coNS3/4A gene had a sequence homology of 79% with the region at nucleotide positions 3417-5475 of the HCV-1 reference strain. A total of 433 nucleotides differed. On an amino acid level the homology with the HCV-1 strain was 98% (15 amino acids differed).

[0271] The full-length codon optimized 2.1 kb DNA fragment of the HCV genotype 1b corresponding to the amino acids 1007 to 1711 encompassing the NS3 and NS4A. NS3/NS4A gene fragment was inserted into a Bam HI and Xba I digested pVAX vector (Invitrogen, San Diego) to give the coNS3/4A-pVAX plasmid. The expression construct was sequenced to ensure correct sequence and reading frame. The protein expression was analysed by an in vitro transcription and translation assay. Plasmids were grown in competent TOP10 E. Coli. (Invitrogen). Plasmid DNA used for in vivo injection, was purified by using Qiagen DNA

purification columns according to the manufacturers instructions (Qiagen GmbH, Hilden, FRG). The concentration of the resulting plasmid DNA was determined spectrophotometrically (Dynaquant, Pharmacia Biotech, Uppsala, Sweden). Purified DNA was dissolved in sterile phosphate buffer saline (PBS) at concentrations of 1 mg/ml.

In vitro translation assay

[0272] To ensure that the wtNS3/4A and coNS3/4A genes were intact and could be translated, an in vitro transcription assay using the prokaryotic T7 coupled reticulocyte lysate system (TNT; Promega, Madison, WI) was performed. To compare the translation efficiency from the two plasmids the amount input DNA was diluted in serial dilutions (6 ng to 1 ng) prior to addition to the TNT assay.

Transient transfections

[0273] HepG2 cells were transiently transfected by standard protocols. In brief, HepG2 cells were plated into 2.5cm² wells (0,5 x 10⁶) in DMEM medium the day before transfection. Two µg of each plasmid DNA construct (wtNS3/4A and coNS3/4A) was transfected into HepG2 cells using Fugene 6 Transfection Reagent (Roche). After transfection, the HepG2 cells were incubated for 24-48hrs.

Protein sample preparation and analysis

[0274] Cell lysates were analysed by immunoprecipitation followed by SDS-PAGE. In brief, transiently transfected HepG2 cells were lysed in RIPA buffer (0,15M NaCl, 50mM Tris, 1% Triton-X 100, 1% Na-deoxycholate and 1% SDS). The cell lysates were immunoprecipitated with protein A sepharose and anti-NS3 polyclonal antibody overnight at 4°C. The washed pellets were re-suspended in SDS sample buffer, heated at 100°C for 5 minutes prior to SDS-PAGE analysis on 4-12% Bis-Tris gel (Invitrogen) and electrotransferred onto Nitrocellulose membranes.

Analysis of NS3 protein expression

[0275] Detection of NS3 protein was done according to manufacturer's protocol by using a chemiluminescence-linked Western blot kit (WesternBreeze; Invitrogen). NS3 protein expression was detected and quantified as a chemiluminescent signal by using an NS3-specific polyclonal antibody. Chemiluminescent signals were detected by using the GeneGnome (Syngene, Cambridge, UK). Quantification of chemiluminescence Western blots was performed

on GeneGnome and units of intensity from each protein band was calculated and compared to a standard curve of rNS3.

Semliki forest virus (SFV) vectors

[0276] Baby Hamster Kidney (BHK)-21 cells were maintained in complete BHK medium supplemented with 5% FCS, 10% tryptose phosphate broth, 2mM glutamine, 20mM Hepes and antibiotics (streptomycin 10µg/ml and penicillin 100 IU/ml).

[0277] The wtNS3/4A gene was isolated by PCR as Spe1-BstB1 fragment and inserted into the Spe1-BstB1 site of pSFV10Enh containing a 34 amino acid long translational enhancer sequence of capsid followed by the FMDV 2a cleavage peptide. Packaging of recombinant RNA into rSFV particles was done using a two-helper RNA system. Indirect immunofluorescence of infected BHK cells was performed to determine the titre of the recombinant virus stocks.

Immuno fluorescence

[0278] BHK cells were transient transfected with coNS3/4A-pVAX1 according to standard techniques using Lipofectamine plus reagent (Invitrogen) or infected by rSFV. NS3 protein was detected by indirect immunofluorescence .

Immunization protocols

[0279] Groups (5-10 mice/group) of female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, 4-8 weeks old, were immunized by needle injections of 100µg of plasmid DNA encoding individual or multiple HCV proteins. Plasmid DNA in PBS was given intramuscularly (i.m.) in the tibialis anterior (TA) muscle. Where indicated in the text, the mice were injected i.m. with 50µL/TA of 0,01mM cardiotoxin (Latoxan, Rosans, France) in 0,9% sterile saline NaCl, five days prior to DNA immunization. The mice were boosted at four-week intervals.

[0280] For gene gun based immunizations, plasmid DNA was linked to gold particles (1µm) according to protocols supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Prior to immunization the abdominal injection area was shaved and the immunization was performed according to the manufacturer's protocol at a helium discharge pressure of 500 psi. Each injection dose contained 4 µg of plasmid DNA. The mice were boosted with the same dose at monthly intervals.

[0281] For rSFV particle immunizations, mice were immunized subcutaneously, in the base of the tail, with 1×10^7 virus particles diluted in PBS (wtNS3/4A-SFV), in a final volume of 100 μ l. Peptide immunization was performed by subcutaneous immunization in the base of the tail with 100 μ g peptide mixed 1:1 in complete Freund's adjuvant.

ELISA for detection of murine anti-HCV NS3 antibodies

[0282] Serum for antibody detection and isotyping was collected every second or fourth week after the first immunization by retroorbital bleeding of isofluorane-anesthetized mice. The enzyme immuno assays were performed as previously described.

Cell lines

[0283] The SP2/0-Ag14 myeloma cell line (H-2^d) was maintained in DMEM medium supplemented with 10% fetal calf serum (FCS; Sigma Chemicals, St. Louis, MO), 2 mM L-Glutamin, 10mM HEPES, 100 U/ml Penicillin and 100 μ g/ml Streptomycin, 1mM non-essential amino acids, 50 μ M β -mercaptoethanol, 1mM sodium pyruvate (GIBCO-BRL, Gaithersburgh, MD). SP2/0-Ag14 cells with stable expression of NS3/4A were maintained in 800 μ g geneticin (G418) /ml complete DMEM medium.

[0284] The EL-4 lymphoma (H-2^b) cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 10mM HEPES, 1mM sodium pyruvate, 1mM non-essential amino acids, 50 μ M β -mercaptoethanol, 100U/ml Penicillin and 100 μ g/ml Streptomycin (GIBCO-BRL). EL-4 cells with stable expression of NS3/4A were generated by transfection of EL-4 cells with the linearized NS3/4A-pcDNA3.1 plasmid using the SuperFect (Qiagen GmbH, Hilden, FRG) transfection reagent. The transfection procedure was performed according to manufacturer's protocol. Transfected cells were cloned by limiting dilution and selected by addition of 800 μ g geneticin (G418) /ml complete RPMI 1640 medium.

[0285] RMA-S cells (a kind gift from Professor Klas Kärre, Karolinska Institutet, Sweden) were maintained in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-Glutamin, 100 U/ml Penicillin and 100 μ g/ml Streptomycin. All cells were grown in a humidified 37°C, 5% CO₂ incubator.

In vivo depletion of T cells

[0286] CD4 and CD8 T cell subpopulations were depleted in vivo by intraperitoneal injection of purified hybridoma supernatant. A total of 0.4 mg per mouse per injection of anti-CD4 (clone GK1.5) or anti-CD8 (clone 53-6.7) was injected on days -3, -2, and -1 before tumor

challenge, and on days 3, 6, 10, and 13 after challenge. Flow cytometric analysis of peripheral blood mononuclear cell populations at days 0, 3, 6, 10, and 13 demonstrated that more than 85% of the CD4 and CD8 T cells were depleted.

In vivo challenge with the NS3/4A-expressing tumor cells

[0287] In vivo challenge of immunized mice with the NS3/4A-expressing SP2/0 myeloma or EL-4 lymphoma cell line was performed according to the method described by Encke et al., supra. In brief, groups of BALB/c or C57BL/6 mice were immunized with different immunogens at weeks zero, four, and eight as described. Two weeks after the last immunisation 1×10^6 NS3/4A-expressing SP2/0 or EL-4 cells were injected subcutaneously in the right flank. The kinetics of the tumor growth was determined by measuring the tumor size through the skin at days six to 20. Kinetic tumor development in two groups of mice was compared using the area under the curve (AUC). The mean tumor sizes were compared using the analysis of variance (ANOVA) test. At day 20 all mice were sacrificed.

[0288] To test the therapeutic effect of the vaccines groups of mice were inoculated with the tumor cells as described above. After six or 12 days the mice were immunized once. The tumor growth was monitored from day 6 to day 20.

Antibodies and MHC:Ig fusion protein

[0289] All monoclonal antibodies and MHC:Ig fusion proteins were purchased from BDB Pharmingen (San Diego, CA); Anti-CD16/CD32 (Fc-block™, clone 2.4G2), FITC conjugated anti-CD8 (clone 53-6.7), Cy-Chrome conjugated anti-CD4 (clone RM4-5), FITC conjugated anti-H-2D^b (clone KH95), recombinant soluble dimeric mouse H-2D^b:Ig, PE conjugated Rat- α Mouse IgG1 (clone X56).

Detection of NS3/4A-specific CTL activity

[0290] Spleen cells from DNA or rSFV immunized C57BL/6 mice were resuspended in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM L-Glutamine, 10mM HEPES, 100 U/ml Penicillin and 100 μ g/ml Streptomycin, 1mM non-essential amino acids, 50 μ M β -mercaptoethanol, 1mM sodium pyruvate. In vitro stimulation was carried out for five days in 25-ml flasks at a final volume of 12 ml, containing 5U/ml recombinant murine IL-2 (mIL-2; R&D Systems, Minneapolis, MN, USA). The restimulation culture contained a total of 25×10^6 immune spleen cells and $2,5 \times 10^6$ irradiated (10,000 rad) syngenic EL-4 cells expressing the NS3/4A protein. After five days in vitro stimulation a

standard ^{51}Cr -release assay was performed. Effector cells were harvested and a four-hour ^{51}Cr assay was performed in 96-well U-bottom plates in a total volume of 200 μl . A total of 1×10^6 target cells (NS3/4A expressing EL-4 cells) was labelled for one hour at $+37^\circ\text{C}$ with 20 μl of ^{51}Cr (5 mCi/ml) and then washed three times in PBS. Different numbers of effectors and ^{51}Cr -labeled target cells (5×10^3 cells/well) were added to wells at effector:target (E:T) ratios of 60:1, 20:1, and 7:1. The level of cytolytic activity was determined after incubation of effectors and targets for 4 hour at $+37^\circ\text{C}$. 100 μl supernatant was harvested and the radioactivity was measured with a γ -counter.

[0291] Splenocytes from DNA or rSFV immunised mice were harvested from C57BL/6 mice and were resuspended in complete RPMI 1640 medium as previously described. In brief, in vitro stimulation was carried out for five days by mixing 25×10^6 spleen cells and 25×10^6 irradiated (2,000 rad) syngeneic splenocytes. The restimulation was performed in the presence of 0,05 μM NS3/4A H-2D^b binding peptide (sequence GAVQNEVTI (Seq. Id. No. 37)). After restimulation, a four hour ^{51}Cr -release assay was performed using ^{51}Cr -labelled peptide pulsed RMA-S cells as targets. Cytotoxic activity was determined at the E:T ratios 60:1, 20:1, and 7:1.

[0292] Results were expressed according to the formula: percent specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release). Experimental release is the mean counts/minute released by the target cells in presence of effector cells. Maximum release is the radioactivity released after lysis of target cells with 10% Triton X-100. Spontaneous release is the leakage of radioactivity into the medium of target cells.

[0293] In vitro T-cell depletion experiments were conducted by incubating effector cells with either an anti-CD4, or anti-CD8, monoclonal antibody containing hybridoma supernatant (clone RL 172.4; anti-CD4, or clone 31M; anti-CD8) for 30 minutes at 4°C . The cells were then washed and incubated at 37°C for 1 hr with complement (1/20 dilution of low toxicity rabbit complement; Saxon, UK) before performing the CTL assay described above.

Quantification of NS3/4A-specific CTLs by flow cytometry

[0294] The frequency of NS3-peptide specific CD8⁺ T cells were analysed by ex-vivo staining of spleen cells from DNA or rSFV immunized mice with recombinant soluble dimeric mouse H-2D^b:Ig fusion protein as previously described. In brief, spleen cells were

resuspended in PBS/1% FCS (FACS buffer) and incubated with Fc-blocking antibodies. Cells were then washed and incubated with H-2D^b:Ig preloaded with NS3/4A derived peptide. Afterwards, cells were washed and incubated with PE conjugated Rat- α Mouse IgG1 antibody, FITC conjugated α -mouse CD8 antibody and Cy-Chrome α -mouse CD4 antibody. After washing, the cells were diluted in FACS buffer containing Propidium Iodide (PI). Approximately 200,000 total events from each sample were acquired on a FACSCalibur (BDB) and dead cells (PI positive cells) were excluded in the analysis.

Statistical analysis

[0295] Fisher's exact test was used for frequency analysis and Mann-Whitney U-test was used for comparing values from two groups. Kinetic tumor development in two groups of mice was compared using the area under the curve (AUC). AUC values were compared using analysis of variance (ANOVA). The calculations were performed using the Macintosh version of the StatView software (version 5.0).

[0296] The compositions described herein may contain other ingredients or compounds in addition to nucleic acids and/or polypeptides, including, but not limited to, various other peptides, adjuvants, binding agents, excipients such as stabilizers (to promote long term storage), emulsifiers, thickening agents, salts, preservatives, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. See e.g., U.S. App. No. 09/929,955 and U.S. App. No. 09/930,591. These compositions are suitable for treatment of animals, particularly mammals, either as a preventive measure to avoid a disease or condition or as a therapeutic to treat animals already afflicted with a disease or condition.

[0297] Many other ingredients may also be present in the compositions provided herein. For example, the adjuvant and antigen can be employed in admixture with conventional excipients (e.g., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral (e.g., oral) or topical application that do not deleteriously react with the adjuvant (e.g., ribavirin) and/or antigen). Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatine, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid,

viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, etc. Many more suitable carriers are described in Remington's Pharmaceutical Sciences, 15th Edition, Easton:Mack Publishing Company, pages 1405-1412 and 1461-1487(1975) and The National Formulary XIV, 14th Edition, Washington, American Pharmaceutical Association (1975).

[0298] The gene constructs described herein, in particular, can be formulated with or administered in conjunction with agents that increase uptake and/or expression of the gene construct by the cells relative to uptake and/or expression of the gene construct by the cells that occurs when the identical genetic vaccine is administered in the absence of such agents. Such agents and the protocols for administering them in conjunction with gene constructs are described in PCT Patent Application Serial Number PCT/US94/00899 filed Jan. 26, 1994. Examples of such agents include: CaPO_4 , DEAE dextran, anionic lipids; extracellular matrix-active enzymes; saponins; lectins; estrogenic compounds and steroidal hormones; hydroxylated lower alkyls; dimethyl sulfoxide (DMSO); urea; and benzoic acid esters anilides, amidines, urethanes and the hydrochloride salts thereof, such as those of the family of local anesthetics. In addition, the gene constructs can be encapsulated within/administered in conjunction with lipids/polycationic complexes.

[0299] Vaccines and immunogenic compositions can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like that do not deleteriously react with the adjuvant or the administered nucleic acid or peptide.

[0300] The effective dose and method of administration of a particular formulation can vary based on the individual patient and the type and stage of the disease, as well as other factors known to those of skill in the art. Therapeutic efficacy and toxicity can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population). The data obtained from cell culture assays and animal studies can be used to formulate a range of dosage for human use. The dosage lies preferably within a range of circulating concentrations that include the ED50 with no toxicity. The dosage varies within this range

depending upon the type of adjuvant derivative and antigen, the dosage form employed, the sensitivity of the patient, and the route of administration.

[0301] Since many adjuvants (e.g., ribavirin) have been on the market for several years, many dosage forms and routes of administration are known. All known dosage forms and routes of administration can be provided within the context of the embodiments described herein. Preferably, an amount of adjuvant that is effective to enhance an immune response to an antigen in an animal can be considered to be an amount that is sufficient to achieve a blood serum level of antigen approximately 0.25 - 12.5µg/ml in the animal, preferably, about 2.5µg/ml. In some embodiments, the amount of adjuvant is determined according to the body weight of the animal to be given the vaccine. Accordingly, the amount of adjuvant in a vaccine formulation can be from about 0.1 - 6.0mg/kg body weight. That is, some embodiments have an amount of adjuvant that corresponds to approximately 0.1 - 1.0mg/kg, 1.1 - 2.0mg/kg, 2.1 - 3.0mg/kg, 3.1 - 4.0mg/kg, 4.1 - 5.0mg/kg, and 5.1 - 6.0mg/kg body weight of an animal. More conventionally, the vaccines contain approximately 0.25mg - 2000mg of adjuvant. That is, some embodiments have approximately 250µg, 500µg, 1mg, 25mg, 50mg, 100mg, 150mg, 200mg, 250mg, 300mg, 350mg, 400mg, 450mg, 500mg, 550mg, 600mg, 650mg, 700mg, 750mg, 800mg, 850mg, 900mg, 1g, 1.1g, 1.2g, 1.3g, 1.4g, 1.5g, 1.6g, 1.7g, 1.8g, 1.9g, and 2g of adjuvant.

[0302] As one of skill in the art will appreciate, the amount of antigens in a vaccine can vary depending on the type of antigen and its immunogenicity. The amount of antigens in the vaccine can vary accordingly. Nevertheless, as a general guide, the vaccines can have approximately 1µg, 5µg, 1µg, 20µg, 40µg, 80µg, 100µg, 0.25mg - 5mg, 5-10mg, 10-100mg, 100-500mg, and upwards of 2000mg of an antigen described herein, for example. Preferably, the amount of antigen is 0.1µg - 1mg, desirably, 0.1µg-100µg, preferably 3µg-50µg, and, most preferably, 7µg, 8µg, 9µg, 10µg, 11µg-20µg, when said antigen is a nucleic acid.

[0303] In some approaches described herein, the exact amount of adjuvant and/or antigen is chosen by the individual physician in view of the patient to be treated. Further, the amounts of adjuvant can be added in combination to or separately from the same or equivalent amount of antigen and these amounts can be adjusted during a particular vaccination protocol so as to provide sufficient levels in light of patient-specific or antigen-

specific considerations. In this vein, patient-specific and antigen-specific factors that can be taken into account include, but are not limited to, the severity of the disease state of the patient, age, and weight of the patient, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy.

Ribavirin

[0304] Nucleoside analogs have been widely used in anti-viral therapies due to their capacity to reduce viral replication. (Hosoya et al., *J. Inf. Dis.*, 168:641-646 (1993)). ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic guanosine analog that has been used to inhibit RNA and DNA virus replication. (Huffman et al., *Antimicrob. Agents. Chemother.*, 3:235 (1973); Sidwell et al., *Science*, 177:705 (1972)). Ribavirin has been shown to be a competitive inhibitor of inositol mono-phosphate (IMP) dehydrogenase (IMPDH), which converts IMP to IMX (which is then converted to GMP). De Clercq, *Anti viral Agents: characteristic activity spectrum depending on the molecular target with which they interact*, Academic press, Inc., New York N.Y., pp. 1-55 (1993). Intracellular pools of GTP become depleted as a result of long term ribavirin treatment.

[0305] In addition to antiviral activity, investigators have observed that some guanosine analogs have an effect on the immune system. (U.S. Patent Nos. 6,063,772 and 4,950,647). Ribavirin has been shown to inhibit functional humoral immune responses (Peavy et al., *J. Immunol.*, 126:861-864 (1981); Powers et al., *Antimicrob. Agents. Chemother.*, 22:108-114 (1982)) and IgE-mediated modulation of mast cell secretion. (Marquardt et al., *J. Pharmacol. Exp. Therapeutics*, 240:145-149 (1987)). Some investigators report that a daily oral therapy of ribavirin has an immune modulating effect on humans and mice. (Hultgren et al., *J. Gen. Virol.*, 79:2381-2391 (1998) and Cramp et al., *Gastron. Enterol.*, 118:346-355 (2000)). Nevertheless, the current understanding of the effects of ribavirin on the immune system is in its infancy. As disclosed below, ribavirin was found to be a potent adjuvant.

EXAMPLE 9

[0306] In a first set of experiments, groups of three to five Balb/c mice (BK Universal, Uppsala, Sweden) were immunized *i.p* or *s.c.* (e.g., at the base of the tail) with 10 μ g or 100 μ g of recombinant hepatitis C virus non-structural 3 (rNS3) protein. The rNS3

was dissolved in phosphate buffered saline (PBS) alone or PBS containing 1mg ribavirin (obtained from ICN, Costa Mesa, CA). Mice were injected with a total volume of 100µl per injection.

[0307] At two and four weeks following *i.p.* immunization, all mice were bled by retro-orbital sampling. Serum samples were collected and analyzed for the presence of antibodies to rNS3. To determine the antibody titer, an enzyme immunoassay (EIA) was performed. (See e.g., Hultgren et al., *J Gen Virol.* 79:2381-91 (1998) and Hultgren et al., *Clin. Diagn. Lab. Immunol.* 4:630-632 (1997)). The antibody levels were recorded as the highest serum dilution giving an optical density at 405nm more than twice that of non-immunized mice.

[0308] Mice that received 10µg or 100µg rNS3 mixed with 1mg ribavirin in PBS displayed consistently higher levels of NS3 antibodies. The antibody titer that was detected by EIA at two weeks post-immunization is shown in **FIGURE 14**. The vaccine formulations having 1mg of ribavirin and either 10µg or 100µg of rNS3 induced a significantly greater antibody titer than the vaccine formulations composed of only rNS3.

[0309] In a second set of experiments, groups of eight Balb/c mice were immunized intraperitoneally with 10 or 50 µg of rNS3 in 100 µl phosphate buffered saline containing either 0 mg, 1 mg, 3 mg, or 10 mg ribavirin (Sigma). At four, six and eight weeks the mice were bled and serum was separated and frozen. After completion of the study, sera were tested for the levels of antibodies to recombinant NS3, as described above. Mean antibody levels to rNS3 were compared between the groups using Student's t-test (parametric analysis) or Mann-Whitney (non-parametric analysis) and the software package StatView 4.5 (Abacus Concepts, Berkely, CA). The adjuvant effect of ribavirin when added in three doses to 10 µg of rNS3 are provided in **TABLE 12**. The adjuvant effect of ribavirin when added in three doses to 50 µg of rNS3 are provided in **TABLE 13**. Parametrical comparison of the mean rNS3 antibody titres in mice receiving different 10µg or 50 µg of rNS3 and different doses of ribavirin are provided in **TABLES 13 and 14**, respectively. Non-parametrical comparison of mean NS3 antibody titres in mice receiving different 10µg or 50 µg of rNS3 and different doses of ribavirin are provided in **TABLES 15-17**, respectively. The values given represent end point titres to recombinant rNS3.

TABLE 12

Amount ribavirin (mg/dose)	Amount immunogen (μ g/dose)	Mouse ID	Antibody titre to rNS3 at indicated week		
			Week 4	Week 6	Week 8
None	10	5:1	300	1500	1500
None	10	5:2	<60	7500	1500
None	10	5:3	<60	1500	300
None	10	5:4	60	1500	1500
None	10	5:5	<60	1500	nt
None	10	5:6	60	1500	1500
None	10	5:7	<60	7500	7500
None	10	5:8	300	37500	7500
Group mean titre (mean $\pm$ SD)			180 $\pm$ 139	7500 $\pm$ 12421	3042 $\pm$ 3076
1	10	6:1	300	37500	37500
1	10	6:2	<60	1500	1500
1	10	6:3	300	37500	187500
1	10	6:4	300	37500	7500
1	10	6:5	60	nt	nt
1	10	6:6	<60	37500	7500
1	10	6:7	<60	37500	7500
1	10	6:8	300	7500	7500
Group mean titre (mean $\pm$ SD)			252 $\pm$ 107	28071 $\pm$ 16195	36642 $\pm$ 67565
3	10	7:1	60	37500	7500
3	10	7:2	60	37500	37500
3	10	7:3	300	7500	7500
3	10	7:4	300	37500	7500
3	10	7:5	300	37500	37500
3	10	7:6	300	37500	37500
3	10	7:7	60	7500	7500
3	10	7:8	60	37500	37500
Group mean titre (mean $\pm$ SD)			180 $\pm$ 128	30000 $\pm$ 13887	22500 $\pm$ 34637
10	10	8:1	300	37500	37500
10	10	8:2	300	37500	37500
10	10	8:3	<60	300	300
10	10	8:4	60	7500	7500
10	10	8:5	<60	300	300
10	10	8:6	<60	37500	37500
10	10	8:7	<60	7500	7500

10	10	8:8	<60	nt	nt
Group mean titre (mean±SD)			220± 139	18300± 18199	18300± 18199

TABLE 13

Amount ribavirin (mg/dose)	Amount immunogen (µg/dose)	Mouse ID	Antibody titre to rNS3 at indicated week		
			Week 4	Week 6	Week 8
None	50	1:1	60	7500	7500
None	50	1:2	60	7500	7500
None	50	1:3	60	7500	7500
None	50	1:4	<60	1500	300
None	50	1:5	300	37500	37500
None	50	1:6	60	7500	7500
None	50	1:7	60	37500	7500
None	50	1:8	.	.	.
Group mean titre (mean±SD)			100 ±98	15214 ±15380	10757 ±12094
1	50	2:1	60	7500	7500
1	50	2:2	300	37500	7500
1	50	2:3	60	187500	7500
1	50	2:4	60	37500	187500
1	50	2:5	60	37500	7500
1	50	2:6	60	37500	37500
1	50	2:7	300	37500	7500
1	50	2:8	300	37500	37500
Group mean titre (mean±SD)			150 ±124	52500 ±55549	37500 ±62105
3	50	3:1	60	37500	7500
3	50	3:2	300	37500	37500
3	50	3:3	300	37500	7500
3	50	3:4	60	37500	7500
3	50	3:5	300	37500	7500
3	50	3:6	60	37500	7500
3	50	3:7	-	7500	37500
3	50	3:8	1500	7500	37500
Group mean titre (mean±SD)			387 ±513	30000 ±13887	18750 ±15526
10	50	4:1	300	7500	7500
10	50	4:2	300	37500	37500
10	50	4:3	60	7500	7500

10	50	4:4	60	7500	7500
10	50	4:5	60	1500	1500
10	50	4:6	60	7500	37500
10	50	4:7	-	7500	7500
10	50	8:8	60	37500	7500
Group mean titre (mean $\pm$ SD)			140 $\pm$ 124	10929 $\pm$ 11928	15214 $\pm$ 15380

TABLE 14

Group	Week	Mean $\pm$ S D	Group	Mean $\pm$ S D	analysis	p-value
10 μ g NS3/no ribavirin	4	180 $\pm$ 139	10 μ g NS3/ 1 mg ribavirin	252 $\pm$ 107	Students t-test	0.4071
	6	7500 $\pm$ 12421		28071 $\pm$ 16195	Students t-test	0.0156*
	8	3042 $\pm$ 3076		36642 $\pm$ 67565	Students t-test	0.2133
10 μ g NS3/no ribavirin	4	180 $\pm$ 139	10 μ g NS3/ 3 mg ribavirin	180 $\pm$ 128	Students t-test	1.000
	6	7500 $\pm$ 12421		30000 $\pm$ 13887	Students t-test	0.0042* *
	8	3042 $\pm$ 3076		22500 $\pm$ 34637	Students t-test	0.0077* *
10 μ g NS3/no ribavirin	4	180 $\pm$ 139	10 μ g NS3/ 10mg ribavirin	220 $\pm$ 139	Students t-test	0.7210
	6	7500 $\pm$ 12421		18300 $\pm$ 18199	Students t-test	0.1974
	8	3042 $\pm$ 3076		18300 $\pm$ 18199	Students t-test	0.0493*

TABLE 15

Group	Week	Mean±S D	Group	Mean±S D	analysis	p-value
50µg NS3/no ribavirin	4	100 ±98	50 µg NS3/ 1 mg ribavirin	150 ±124	Students t-test	0.4326
	6	15214 ±15380		52500 ±55549	Students t-test	0.1106
	8	10757 ±12094		37500 ±62105	Students t-test	0.2847
50µg NS3/no ribavirin	4	100 ±98	50 µg NS3/ 3 mg ribavirin	387 ±513	Students t-test	0.2355
	6	15214 ±15380		30000 ±13887	Students t-test	0.0721
	8	10757 ±12094		18750 ±15526	Students t-test	0.2915
50µg NS3/no ribavirin	4	100 ±98	50 µg NS3/ 10mg ribavirin	140 ±124	Students t-test	0.5490
	6	15214 ±15380		10929 ±11928	Students t-test	0.5710
	8	10757 ±12094		15214 ±15380	Students t-test	0.5579

Significance levels: NS = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001

TABLE 16

Group	Week	Mean±S D	Group	Mean±S D	analysis	p-value
10µg NS3/no ribavirin	4	180 ±139	10 µg NS3/ 1 mg ribavirin	252 ±107	Mann- Whitney	0.4280
	6	7500 ±12421		28071 ±16195	Mann- Whitney	0.0253*
	8	3042 ±3076		36642 ±67565	Mann- Whitney	0.0245*
10µg NS3/no ribavirin	4	180 ±139	10 µg NS3/ 3 mg ribavirin	180± 128	Mann- Whitney	0.0736

	6	7500 ±12421		30000± 13887	Mann- Whitney	0.0050* *
	8	3042 ±3076		22500± 34637	Mann- Whitney	0.0034* *
10µg NS3/no ribavirin	4	180 ±139	10 µg NS3/ 10mg ribavirin	220± 139	Mann- Whitney	0.8986
	6	7500 ±12421		18300± 18199	Mann- Whitney	0.4346
	8	3042 ±3076		18300± 18199	Mann- Whitney	0.2102

TABLE 17

Group	Week	Mean±S D	Group	Mean±S D	analysis	p-value
50µg NS3/no ribavirin	4	100 ±98	50 µg NS3/ 1 mg ribavirin	150 ±124	Mann- Whitney	0.1128
	6	15214 ±15380		52500 ±55549	Mann- Whitney	0.0210*
	8	10757 ±12094		37500 ±62105	Mann- Whitney	0.1883
50µg NS3/no ribavirin	4	100 ±98	50 µg NS3/ 3 mg ribavirin	387 ±513	Mann- Whitney	0.1400
	6	15214 ±15380		30000 ±13887	Mann- Whitney	0.0679
	8	10757 ±12094		18750 ±15526	Mann- Whitney	0.2091
50µg NS3/no ribavirin	4	100 ±98	50 µg NS3/ 10 mg ribavirin	140 ±124	Mann- Whitney	0.4292
	6	15214 ±15380		10929 ±11928	Mann- Whitney	0.9473
	8	10757 ±12094		15214 ±15380	Mann- Whitney	0.6279

Significance levels: NS = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001

I.

The data above demonstrates that ribavirin facilitates or enhances an immune response to an HCV antigen or HCV epitopes. A potent immune response to rNS3 was elicited after immunization with a vaccine composition comprising as little as 1 mg ribavirin and 10 µg of rNS3 antigen. The data above also provide evidence that the amount of ribavirin that is sufficient to facilitate an immune response to an antigen is between 1 and 3 mg per injection for a 25-30g Balb/c mouse. It should be realized, however, that these amounts are intended for guidance only and should not be interpreted to limit the scope of the invention in any way. Nevertheless, the data shows that vaccine compositions comprising approximately 1 to 3 mg doses of ribavirin induce an immune response that is more than 12 times higher than the immune response elicited in the absence of without ribavirin. Thus, ribavirin has a significant adjuvant effect on the humoral immune response of an animal and thereby, enhances or facilitates the immune response to the antigen. The example below describes experiments that were performed to better understand the amount of ribavirin needed to enhance or facilitate an immune response to an antigen.

EXAMPLE 10

[0310] To determine a dose of ribavirin that is sufficient to provide an adjuvant effect, the following experiments were performed. In a first set of experiments, groups of mice (three per group) were immunized with a 20µg rNS3 alone or a mixture of 20µg rNS3 and 0.1mg, 1mg, or 10mg ribavirin. The levels of antibody to the antigen were then determined by EIA. The mean endpoint titers at weeks 1 and 3 were plotted and are shown in **FIGURE 15**. It was discovered that the adjuvant effect provided by ribavirin had different kinetics depending on the dose of ribavirin provided. For example, even low doses (<1mg) of ribavirin were found to enhance antibody levels at week one but not at week three, whereas, higher doses (1-10mg) were found to enhance antibody levels at week three.

[0311] A second set of experiments was also performed. In these experiments, groups of mice were injected with vaccine compositions comprising various amounts of ribavirin and rNS3 and the IgG response in these animals was monitored. The vaccine compositions comprised approximately 100 µl phosphate buffered saline and 20 µg rNS3 with or without 0.1 mg, 1.0 mg, or 10 mg ribavirin (Sigma). The mice were bled at week six and rNS3-specific IgG levels were determined by EIA as described previously. As shown in

TABLE 18, the adjuvant effects on the sustained antibody levels were most obvious in the dose range of 1 to 10 mg per injection for a 25-30g mouse.

TABLE 18

Immunogen	Amount (mg) ribavirin mixed with the immunogen	Mouse ID	Endpoint titre of rNS3 IgG at indicated week		
			Week 1	Week 2	Week 3
20 µg rNS3	None	1	60	360	360
20 µg rNS3	None	2	360	360	2160
20 µg rNS3	None	3	360	2160	2160
		Mean	260±173	960±1039	1560±1039
20 µg rNS3	0.1	4	2160	12960	2160
20 µg rNS3	0.1	5	60	60	60
20 µg rNS3	0.1	6	<60	2160	2160
			1110±1484	5060±6921	1460±1212
20 µg rNS3	1.0	7	<60	60	12960
20 µg rNS3	1.0	8	<60	2160	2160
20 µg rNS3	1.0	9	360	2160	2160
		Mean	360	1460±1212	5760±6235
20 µg rNS3	10.0	10	360	12960	77760
20 µg rNS3	10.0	11	<60	2160	12960
20 µg rNS3	10.0	12	360	2160	2160
		Mean	360	5760±6235	30960±40888

[0312] In a third set of experiments, the adjuvant effect of ribavirin after primary and booster injections was investigated. In these experiments, mice were given two intraperitoneal injections of a vaccine composition comprising 10 µg rNS3 with or without ribavirin and the IgG subclass responses to the antigen was monitored, as before. Accordingly, mice were immunized with 100 µl phosphate buffered containing 10 µg recombinant NS3 alone, with or without 0.1 or 1.0 mg ribavirin (Sigma) at weeks 0 and 4. The mice were bled at week six and NS3-specific IgG subclasses were determined by EIA as described previously. As shown in **TABLE 21**, the addition of ribavirin to the immunogen prior to the injection does not change the IgG subclass response in the NS3-specific immune

response. Thus, the adjuvant effect of a vaccine composition comprising ribavirin and an antigen can not be explained by a shift in of the Th1/Th2-balance. It appears that another mechanism may be responsible for the adjuvant effect of ribavirin.

TABLE 19

Immunogen	Amount (mg) ribavirin mixed with the immunogen	Mouse ID	Endpoint titre of indicated NS3 IgG subclass			
			IgG1	IgG2a	IgG2b	IgG3
10 µg rNS3	None	1	360	60	<60	60
10 µg rNS3	None	2	360	<60	<60	60
10 µg rNS3	None	3	2160	60	<60	360
		Mean	960±1039	60	-	160±173
10 µg rNS3	0.1	4	360	<60	<60	60
10 µg rNS3	0.1	5	60	<60	<60	<60
10 µg rNS3	0.1	6	2160	60	60	360
			860±1136	60	60	210±212
10 µg rNS3	1.0	7	2160	<60	<60	60
10 µg rNS3	1.0	8	360	<60	<60	<60
10 µg rNS3	1.0	9	2160	<60	<60	60
		Mean	1560±1039	-	-	60

[0313] The data presented in this example further verify that ribavirin can be administered as an adjuvant and establish that that the dose of ribavirin can modulate the kinetics of the adjuvant effect. The example below describes another assay that was performed to evaluate the ability of ribavirin to enhance or facilitate an immune response to an antigen.

EXAMPLE 11

[0314] This assay can be used with any ribavirin derivative or combinations of ribavirin derivatives to determine the extent that a particular vaccine formulation modulates a cellular immune response. To determine CD4⁺ T cell responses to a ribavirin-containing vaccine, groups of mice were immunized *s.c.* with either 100µg rNS3 in PBS or 100µg rNS3 and 1mg ribavirin in PBS. The mice were sacrificed ten days post-immunization and their

lymph nodes were harvested and drained. *In vitro* recall assays were then performed. (See e.g., Hultgren et al., *J Gen Virol.* 79:2381-91 (1998) and Hultgren et al., *Clin. Diagn. Lab. Immunol.* 4:630-632 (1997)). The amount of CD4⁺ T cell proliferation was determined at 96 h of culture by the incorporation of [³H] thymidine.

[0315] As shown in **FIGURE 16**, mice that were immunized with 100µg rNS3 mixed with 1mg ribavirin had a much greater T cell proliferative response than mice that were immunized with 100µg rNS3 in PBS. This data provides more evidence that ribavirin enhances or facilitates a cellular immune response (e.g., by promoting the effective priming of T cells).

[0316] Additional experiments were conducted to verify that ribavirin enhances the immune response to commercially available vaccine preparations. The example below describes the use of ribavirin in conjunction with a commercial HBV vaccine preparation.

EXAMPLE 12

[0317] The adjuvant effect of ribavirin was tested when mixed with two doses of a commercially available vaccine containing HBsAg and alum. (Engerix, SKB). Approximately 0.2µg or 2µg of Engerix vaccine was mixed with either PBS or 1mg ribavirin in PBS and the mixtures were injected intra peritoneally into groups of mice (three per group). A booster containing the same mixture was given on week four and all mice were bled on week six. The serum samples were diluted from 1:60 to 1:37500 and the dilutions were tested by EIA, as described above, except that purified human HBsAg was used as the solid phase antigen. As shown in **TABLE 20**, vaccine formulations having ribavirin enhanced the response to 2µg of an existing vaccine despite the fact that the vaccine already contained alum. That is, by adding ribavirin to a suboptimal vaccine dose (i.e., one that does not induce detectable antibodies alone) antibodies became detectable, providing evidence that the addition of ribavirin allows for the use of lower antigen amounts in a vaccine formulation without compromising the immune response.

TABLE 20

Week	End point antibody titer to HBsAg in EIA											
	0.02 μ g Engerix						0.2 μ g Engerix					
	No ribavirin			1mg ribavirin			No ribavirin			1mg ribavirin		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
6	<60	<60	<60	<60	<60	<60	<60	<60	<60	300	60	<60

[0318] The ribavirin used in the experiments above was obtained from commercial suppliers (e.g., Sigma and ICN). The ribavirin that can be used with the embodiments described herein can also be obtained from commercial suppliers or can be synthesized. The ribavirin and/or the antigen can be formulated with and without modification. For example, the ribavirin can be modified or derivatized to make a more stable molecule and/or a more potent adjuvant. By one approach, the stability of ribavirin can be enhanced by coupling the molecules to a support such as a hydrophilic polymer (e.g., polyethylene glycol).

[0319] Many more ribavirin derivatives can be generated using conventional techniques in rational drug design and combinatorial chemistry. For example, Molecular Simulations Inc. (MSI), as well as many other suppliers, provide software that allows one of skill to build a combinatorial library of organic molecules. The C2.Analog Builder program, for example, can be integrated with MSI's suite of Cerius2 molecular diversity software to develop a library of ribavirin derivatives that can be used with the embodiments described herein. (See e.g., <http://msi.com/life/products/cerius2/index.html>).

[0320] By one approach, the chemical structure of ribavirin is recorded on a computer readable media and is accessed by one or more modeling software application programs. The C2.Analog Builder program in conjunction with C2Diversity program allows the user to generate a very large virtual library based on the diversity of R-groups for each substituent position, for example. Compounds having the same structure as the modeled ribavirin derivatives created in the virtual library are then made using conventional chemistry or can be obtained from a commercial source.

[0321] The newly manufactured ribavirin derivatives can then be screened in assays, which determine the extent of adjuvant activity of the molecule and/or the extent of

its ability to modulate of an immune response. Some assays may involve virtual drug screening software, such as C2.Ludi. C2.Ludi is a software program that allows a user to explore databases of molecules (e.g., ribavirin derivatives) for their ability to interact with the active site of a protein of interest (e.g., RAC2 or another GTP binding protein). Based upon predicted interactions discovered with the virtual drug screening software, the ribavirin derivatives can be prioritized for further characterization in conventional assays that determine adjuvant activity and/or the extent of a molecule to modulate an immune response. The section below provides more explanation concerning the methods of using the compositions described herein.

Methods of using the vaccine compositions and immunogen preparations

[0322] Routes of administration of the embodiments described herein include, but are not limited to, transdermal, parenteral, gastrointestinal, transbronchial, and transalveolar. Transdermal administration can be accomplished by application of a cream, rinse, gel, etc. capable of allowing the adjuvant and HCV antigen to penetrate the skin. Parenteral routes of administration include, but are not limited to, electrical or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. Transbronchial and transalveolar routes of administration include, but are not limited to, inhalation, either via the mouth or intranasally.

[0323] Compositions having the adjuvant and HCV antigen that are suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams, and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device ("transdermal patch"). Examples of suitable creams, ointments, etc. can be found, for instance, in the Physician's Desk Reference. Examples of suitable transdermal devices are described, for instance, in U.S. Patent No. 4,818,540 issued April 4, 1989 to Chinen, et al.

[0324] Compositions having the adjuvant and HCV antigen that are suitable for parenteral administration include, but are not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline, phosphate buffered saline and oil preparations for injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection.

[0325] Compositions having the adjuvant and HCV antigen that are suitable for transbronchial and transalveolar administration include, but not limited to, various types of aerosols for inhalation. Devices suitable for transbronchial and transalveolar administration of these are also embodiments. Such devices include, but are not limited to, atomizers and vaporizers. Many forms of currently available atomizers and vaporizers can be readily adapted to deliver vaccines having ribavirin and an antigen.

[0326] Compositions having the adjuvant and HCV antigen that are suitable for gastrointestinal administration include, but not limited to, pharmaceutically acceptable powders, pills or liquids for ingestion and suppositories for rectal administration.

[0327] The gene constructs described herein, in particular, may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Alternatively, the genetic vaccine may be introduced by various means into cells that are removed from the individual. Such means include, for example, *ex vivo* transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated therein can be implanted into the individual even if the vaccinated cells were originally taken from another individual.

[0328] According to some embodiments, the gene construct is administered to an individual using a needleless injection device. According to some embodiments, the gene construct is simultaneously administered to an individual intradermally, subcutaneously and intramuscularly using a needleless injection device. Needleless injection devices are well known and widely available. One having ordinary skill in the art can, following the teachings herein, use needleless injection devices to deliver genetic material to cells of an individual. Needleless injection devices are well suited to deliver genetic material to all tissue. They are particularly useful to deliver genetic material to skin and muscle cells. In some embodiments, a needleless injection device may be used to propel a liquid that contains DNA molecules toward the surface of the individual's skin. The liquid is propelled at a sufficient velocity such that upon impact with the skin the liquid penetrates the surface of the skin, permeates the skin and muscle tissue therebeneath. Thus, the genetic material is simultaneously administered intradermally, subcutaneously and intramuscularly. In some

embodiments, a needleless injection device may be used to deliver genetic material to tissue of other organs in order to introduce a nucleic acid molecule to cells of that organ.

[0329] Preferred embodiments concern methods of treating or preventing HCV infection. In these embodiments, an animal in need is provided an HCV antigen (e.g., a peptide antigen or nucleic acid-based antigen, as described herein (**SEQ. ID. NOs.: 1-27, 35-36, and 40-220**) (including wild-type and codon optimized sequences encoding **SEQ ID NOs: 40-220**) and an amount of adjuvant sufficient to exhibit an adjuvant activity in said animal. Accordingly, an animal can be identified as one in need by using currently available diagnostic testing or clinical evaluation. The adjuvant and antigen can be provided separately or in combination, and other adjuvants (e.g., oil, alum, or other agents that enhance an immune response) can also be provided to the animal in need.

[0330] Other embodiments of the invention include methods of enhancing an immune response to an HCV antigen by providing an animal in need with an amount of adjuvant (e.g., ribavirin) and one or more of **SEQ. ID. NOs.: 1-11, 35-36, and 40-220** (or a wild type or codon-optimized nucleic acid encoding **SEQ ID NOs: 40-220**) or a fragment thereof, preferably **SEQ. ID. NOs.: 12-27** that is effective to enhance said immune response. In these embodiments, an animal in need of an enhanced immune response to an antigen is identified by using currently available diagnostic testing or clinical evaluation. By one approach, for example, an uninfected individual is provided with the vaccine compositions described above in an amount sufficient to elicit a cellular and humoral immune response to NS3 so as to protect said individual from becoming infected with HCV. In another embodiment, an HCV-infected individual is identified and provided with a vaccine composition comprising ribavirin and NS3 in an amount sufficient to enhance the cellular and humoral immune response against NS3 so as to reduce or eliminate the HCV infection. Such individual may be in the chronic or acute phase of the infection. In yet another embodiment, an HCV-infected individual suffering from HCC is provided with a composition comprising an adjuvant and the NS3/4A fusion gene in an amount sufficient to elicit a cellular and humoral immune response against NS3-expressing tumor cells.

[0331] The next section describes some of the peptide embodiments of the invention.

HCV Peptides

[0332] The embodied HCV peptides or derivatives thereof, include but are not limited to, those containing as a primary amino acid sequence all of the amino acid sequence substantially as depicted in the Sequence Listing (**SEQ. ID. NOs.: 2-11, 36, and SEQ ID NOs: 40-220**) and fragments of **SEQ. ID. NOs.: 2-11** and **SEQ. ID. NO.: 36** that are at least four amino acids in length (e.g., **SEQ. ID. NOs.: 14-16**) including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. Preferred fragments of a sequence of **SEQ. ID. NOs.: 2-11** and **SEQ. ID. NO.: 36** are at least four amino acids and comprise amino acid sequence unique to the discovered NS3/4A peptide or mutants thereof including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. The HCV peptides can be, for example, at least 12-704 amino acids in length (e.g., any number between 12-15, 15-20, 20-25, 25-50, 50-100, 100-150, 150-250, 250-500 or 500-704 amino acids in length).

[0333] Embodiments also include HCV peptides that are substantially identical to those described above. That is, HCV peptides that have one or more amino acid residues within **SEQ. ID. NOs.: 2-11, 36, and 40-220** and fragments thereof that are substituted by another amino acid of a similar polarity that acts as a functional equivalent, resulting in a silent alteration. Further, the HCV peptides can have one or more amino acid residues fused to **SEQ. ID. NOs.: 2-11, 36** and **SEQ ID NO: 40-220** or a fragment thereof so long as the fusion does not significantly alter the structure or function (e.g., immunogenic properties) of the HCV peptide. Substitutes for an amino acid within the sequence can be selected from other members of the class to which the amino acid belongs. For example, the non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. The aromatic amino acids include phenylalanine, tryptophan, and tyrosine. Accordingly, the peptide embodiments of the invention are said to be consisting essentially of **SEQ. ID. NOs.: 2-27, 36** and **SEQ ID NOs: 40-220** in light of the modifications described above.

[0334] The HCV peptides described herein can be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using techniques known in the art such as those set forth by Merrifield et al., *J. Am. Chem. Soc.* 85:2149 (1964), Houghten et al., *Proc. Natl. Acad. Sci. USA*, 82:51:32 (1985), Stewart and Young (Solid phase peptide synthesis, Pierce Chem Co., Rockford, IL (1984), and Creighton, 1983, Proteins: Structures and Molecular Principles, W. H. Freeman & Co., N.Y. Such polypeptides can be synthesized with or without a methionine on the amino terminus. Chemically synthesized HCV peptides can be oxidized using methods set forth in these references to form disulfide bridges.

[0335] While the HCV peptides described herein can be chemically synthesized, it can be more effective to produce these polypeptides by recombinant DNA technology. Such methods can be used to construct expression vectors containing the HCV nucleotide sequences described above, for example, and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Alternatively, RNA capable of encoding an HCV nucleotide sequence can be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in Oligonucleotide Synthesis, 1984, Gait, M. J. ed., IRL Press, Oxford. Accordingly, several embodiments concern cell lines that have been engineered to express the embodied HCV peptides. For example, some cells are made to express the HCV peptides of **SEQ. ID. NOs.: 2-11, 36** and **SEQ ID NOs: 40-220** or fragments of these molecules (e.g., **SEQ. ID. NOs.: 14-26**).

[0336] A variety of host-expression vector systems can be utilized to express the embodied HCV peptides. Suitable expression systems include, but are not limited to, microorganisms such as bacteria (e.g., *E. coli* or *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing HCV nucleotide sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the HCV nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the HCV sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing HCV sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant

expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[0337] In bacterial systems, a number of expression vectors can be advantageously selected depending upon the use intended for the HCV gene product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of HCV peptide or for raising antibodies to the HCV peptide, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified can be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., *EMBO J.*, 2:1791 (1983), in which the HCV coding sequence can be ligated individually into the vector in frame with the *lacZ* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.*, 13:3101-3109 (1985); Van Heeke & Schuster, *J. Biol. Chem.*, 264:5503-5509 (1989)); and the like. The pGEX vectors can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[0338] In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The HCV coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of an HCV gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus, (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (See e.g., Smith et al., *J. Virol.* 46: 584 (1983); and Smith, U.S. Pat. No. 4,215,051).

[0339] In mammalian host cells, a number of viral-based expression systems can be utilized. In cases where an adenovirus is used as an expression vector, the HCV

nucleotide sequence of interest can be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the HCV gene product in infected hosts. (See e.g., Logan & Shenk, *Proc. Natl. Acad. Sci. USA* 81:3655-3659 (1984)). Specific initiation signals can also be required for efficient translation of inserted HCV nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences.

[0340] However, in cases where only a portion of the HCV coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, can be provided. Furthermore, the initiation codon can be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., *Methods in Enzymol.*, 153:516-544 (1987)).

[0341] In addition, a host cell strain can be chosen, which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products are important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

[0342] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the HCV peptides described above can be engineered. Rather than using expression vectors that contain viral

origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells are allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn are cloned and expanded into cell lines. This method is advantageously used to engineer cell lines which express the HCV gene product.

[0343] A number of selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., *Cell* 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* 48:2026 (1962)), and adenine phosphoribosyltransferase (Lowy, et al., *Cell* 22:817 (1980)) genes can be employed in *tk⁻*, *hgpri⁻* or *apri⁻* cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: *dhfr*, which confers resistance to methotrexate (Wigler, et al., *Proc. Natl. Acad. Sci. USA* 77:3567 (1980); O'Hare, et al., *Proc. Natl. Acad. Sci. USA* 78:1527 (1981)); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072 (1981)); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., *J. Mol. Biol.* 150:1 (1981)); and *hygro*, which confers resistance to hygromycin (Santerre, et al., *Gene* 30:147 (1984)).

[0344] Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. (Janknecht, et al., *Proc. Natl. Acad. Sci. USA* 88: 8972-8976 (1991)). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni²⁺ nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers. The example below describes a method that was used to express the HCV peptides encoded by the embodied nucleic acids.

EXAMPLE 13

[0345] To characterize NS3/4A-pVAX, MSLF1-pVAX, and the NS3/4A mutant constructs, described in *Example 1*, the plasmids were transcribed and translated *in vitro*, and the resulting polypeptides were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *In vitro* transcription and translation were performed using the T7 coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions. All *in vitro* translation reactions of the expression constructs were carried out at 30°C with ³⁵S-labeled methionine (Amersham International, Plc, Buckinghamshire, UK). The labeled proteins were separated by 12% SDS-PAGE and visualized by exposure to X-ray film (Hyper Film-MP, Amersham) for 6-18 hours.

[0346] The *in vitro* analysis revealed that all proteins were expressed to high amounts from their respective expression constructs. The rNS3 construct (NS3-pVAX vector) produced a single peptide of approximately 61kDa, whereas, the mutant constructs (e.g., the TGT construct (NS3/4A-TGT-pVAX) and the RGT construct (NS3/4A-RGT-pVAX)) produced a single polypeptide of approximately 67 kDa, which is identical to the molecular weight of the uncleaved NS3/4A peptide produced from the NS3/4A-pVAX construct. The cleaved product produced from the expressed NS3/4A peptide was approximately 61 kDa, which was identical in size to the rNS3 produced from the NS3-pVAX vector. These results demonstrated that the expression constructs were functional, the NS3/4A construct was enzymatically active, the rNS3 produced a peptide of the predicted size, and the breakpoint mutations completely abolished cleavage at the NS3-NS4A junction.

[0347] To compare the translation efficiency from the NS3/4A-pVAX and MSLF1-pVAX plasmids, the amount of input DNA was serially diluted prior to addition to the assay. Serial dilutions of the plasmids revealed that the MSLF1 plasmid gave stronger bands at higher dilutions of the plasmid than the wild-type NS3/4A plasmid, providing evidence that *in vitro* transcription and translation was more efficient from the MSLF1 plasmid. The NS3/4A-pVAX and MSLF1 plasmids were then analyzed for protein expression using transiently transfected Hep-G2 cells. Similar results were obtained in that the MSLF-1 gene provided more efficient expression of NS3 than the native NS3/4A gene.

[0348] The sequences, constructs, vectors, clones, and other materials comprising the embodied HCV nucleic acids and peptides can be in enriched or isolated form. As used herein, “enriched” means that the concentration of the material is many times its natural concentration, for example, at least about 2, 5, 10, 100, or 1000 times its natural concentration, advantageously 0.01%, by weight, preferably at least about 0.1% by weight. Enriched preparations from about 0.5% or more, for example, 1%, 5%, 10%, and 20% by weight are also contemplated. The term “isolated” requires that the material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide present in a living animal is not isolated, but the same polynucleotide, separated from some or all of the coexisting materials in the natural system, is isolated. It is also advantageous that the sequences be in purified form. The term “purified” does not require absolute purity; rather, it is intended as a relative definition. Isolated proteins have been conventionally purified to electrophoretic homogeneity by Coomassie staining, for example. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

[0349] The HCV gene products described herein can also be expressed in plants, insects, and animals so as to create a transgenic organism. Desirable transgenic plant systems having an HCV peptide include *Arabidopsis*, maize, and *Chlamydomonas*. Desirable insect systems having an HCV peptide include, but are not limited to, *D. melanogaster* and *C. elegans*. Animals of any species, including, but not limited to, amphibians, reptiles, birds, mice, hamsters, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, dogs, cats, and non-human primates, e.g., baboons, monkeys, and chimpanzees can be used to generate transgenic animals having an embodied HCV molecule. These transgenic organisms desirably exhibit germline transfer of HCV peptides described herein.

[0350] Any technique known in the art is preferably used to introduce the HCV transgene into animals to produce the founder lines of transgenic animals or to knock out or replace existing HCV genes. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P. C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., *Proc. Natl. Acad. Sci., USA* 82:6148-6152 (1985)); gene targeting in embryonic stem cells (Thompson et al., *Cell* 56:313-

321 (1989)); electroporation of embryos (Lo, *Mol Cell. Biol.* 3:1803-1814 (1983); and sperm-mediated gene transfer (Lavitrano et al., *Cell* 57:717-723 (1989)); see also Gordon, *Transgenic Animals, Intl. Rev. Cytol.* 115:171-229 (1989).

[0351] Following synthesis or expression and isolation or purification of the HCV peptides, the isolated or purified peptide can be used to generate antibodies. Depending on the context, the term "antibodies" can encompass polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library. Antibodies that recognize the HCV peptides have many uses including, but not limited to, biotechnological applications, therapeutic/prophylactic applications, and diagnostic applications.

[0352] For the production of antibodies, various hosts including goats, rabbits, rats, mice, and humans etc. can be immunized by injection with an HCV peptide. Depending on the host species, various adjuvants can be used to increase immunological response. Such adjuvants include, but are not limited to, ribavirin, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacillus Calmette-Guerin*) and *Corynebacterium parvum* are also potentially useful adjuvants.

[0353] Peptides used to induce specific antibodies can have an amino acid sequence consisting of at least four amino acids, and preferably at least 10 to 15 amino acids. By one approach, short stretches of amino acids encoding fragments of NS3/4A are fused with those of another protein such as keyhole limpet hemocyanin such that an antibody is produced against the chimeric molecule. Additionally, a composition comprising ribavirin and an HCV peptide (**SEQ. ID. NOs.: 2-11, 40-220** and **SEQ. ID. NO.: 36**), a fragment thereof containing any number of consecutive amino acids between at least 3-50 (e.g., 3, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids) (e.g., **SEQ. ID. NOs.: 4-26**), or a nucleic acid encoding one or more of these molecules is administered to an animal, preferably a mammal including a human. While antibodies capable of specifically recognizing HCV can be generated by injecting synthetic 3-mer, 10-mer, and 15-mer peptides that correspond to an HCV peptide into mice, a more diverse set of antibodies can be generated by using recombinant HCV peptides, prepared as described above.

[0354] To generate antibodies to an HCV peptide, substantially pure peptide is isolated from a transfected or transformed cell. The concentration of the peptide in the final

preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the peptide of interest can then be prepared as follows:

[0355] Monoclonal antibodies to an HCV peptide can be prepared using any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (*Nature* 256:495-497 (1975)), the human B-cell hybridoma technique (Kosbor et al. *Immunol Today* 4:72 (1983)); Cote et al *Proc Natl Acad Sci* 80:2026-2030 (1983), and the EBV-hybridoma technique Cole et al. Monoclonal Antibodies and Cancer Therapy, Alan R. Liss Inc, New York N.Y., pp 77-96 (1985). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used. (Morrison et al. *Proc Natl Acad Sci* 81:6851-6855 (1984); Neuberger et al. *Nature* 312:604-608(1984); Takeda et al. *Nature* 314:452-454(1985)). Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce HCV-specific single chain antibodies. Antibodies can also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi et al., *Proc Natl Acad Sci* 86: 3833-3837 (1989), and Winter G. and Milstein C; *Nature* 349:293-299 (1991).

[0356] Antibody fragments that contain specific binding sites for an HCV peptide can also be generated. For example, such fragments include, but are not limited to, the F(ab')₂ fragments that can be produced by pepsin digestion of the antibody molecule and the Fab fragments that can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries can be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (Huse W. D. et al. *Science* 256:1275-1281 (1989)).

[0357] By one approach, monoclonal antibodies to an HCV peptide are made as follows. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein or peptides derived therefrom over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused in

the presence of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall, E., *Meth. Enzymol.* 70:419 (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. et al. Basic Methods in Molecular Biology Elsevier, New York. Section 21-2.

[0358] Polyclonal antiserum containing antibodies to heterogeneous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein or peptides derived therefrom described above, which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and can require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. An effective immunization protocol for rabbits can be found in Vaitukaitis, J. et al. *J. Clin. Endocrinol. Metab.* 33:988-991 (1971).

[0359] Booster injections are given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. et al., Chap. 19 in: Handbook of Experimental Immunology D. Wier (ed) Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 μ M). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: Manual of Clinical Immunology, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D.C. (1980). Antibody preparations prepared according to either protocol are useful in quantitative immunoassays that determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively (e.g., in

diagnostic embodiments that identify the presence of HCV in biological samples). The next section describes how some of the novel nucleic acids and peptides described above can be used in diagnostics.

Diagnostic Embodiments

[0360] Generally, the embodied diagnostics are classified according to whether a nucleic acid or protein-based assay is used. Some diagnostic assays detect the presence or absence of an embodied HCV nucleic acid sequence in a sample obtained from a patient, whereas, other assays seek to identify whether an embodied HCV peptide is present in a biological sample obtained from a patient. Additionally, the manufacture of kits that incorporate the reagents and methods described herein that allow for the rapid detection and identification of HCV are also embodied. These diagnostic kits can include, for example, an embodied nucleic acid probe or antibody, which specifically detects HCV. The detection component of these kits will typically be supplied in combination with one or more of the following reagents. A support capable of absorbing or otherwise binding DNA, RNA, or protein will often be supplied. Available supports include membranes of nitrocellulose, nylon or derivatized nylon that can be characterized by bearing an array of positively charged substituents. One or more restriction enzymes, control reagents, buffers, amplification enzymes, and non-human polynucleotides like calf-thymus or salmon-sperm DNA can be supplied in these kits.

[0361] Useful nucleic acid-based diagnostics include, but are not limited to, direct DNA sequencing, Southern Blot analysis, dot blot analysis, nucleic acid amplification, and combinations of these approaches. The starting point for these analysis is isolated or purified nucleic acid from a biological sample obtained from a patient suspected of contracting HCV or a patient at risk of contracting HCV. The nucleic acid is extracted from the sample and can be amplified by RT-PCR and/or DNA amplification using primers that correspond to regions flanking the embodied HCV nucleic acid sequences (e.g., NS3/4A (**SEQ. ID. NO.:** 1)).

[0362] In some embodiments, nucleic acid probes that specifically hybridize with HCV sequences are attached to a support in an ordered array, wherein the nucleic acid probes are attached to distinct regions of the support that do not overlap with each other. Preferably, such an ordered array is designed to be "addressable" where the distinct locations of the

probe are recorded and can be accessed as part of an assay procedure. These probes are joined to a support in different known locations. The knowledge of the precise location of each nucleic acid probe makes these "addressable" arrays particularly useful in binding assays. The nucleic acids from a preparation of several biological samples are then labeled by conventional approaches (e.g., radioactivity or fluorescence) and the labeled samples are applied to the array under conditions that permit hybridization.

[0363] If a nucleic acid in the samples hybridizes to a probe on the array, then a signal will be detected at a position on the support that corresponds to the location of the hybrid. Since the identity of each labeled sample is known and the region of the support on which the labeled sample was applied is known, an identification of the presence of the polymorphic variant can be rapidly determined. These approaches are easily automated using technology known to those of skill in the art of high throughput diagnostic or detection analysis.

[0364] Additionally, an approach opposite to that presented above can be employed. Nucleic acids present in biological samples can be disposed on a support so as to create an addressable array. Preferably, the samples are disposed on the support at known positions that do not overlap. The presence of HCV nucleic acids in each sample is determined by applying labeled nucleic acid probes that complement nucleic acids, which encode HCV peptides, at locations on the array that correspond to the positions at which the biological samples were disposed. Because the identity of the biological sample and its position on the array is known, the identification of a patient that has been infected with HCV can be rapidly determined. These approaches are also easily automated using technology known to those of skill in the art of high throughput diagnostic analysis.

[0365] Any addressable array technology known in the art can be employed. One particular embodiment of polynucleotide arrays is known as Genechips™, and has been generally described in US Patent 5,143,854; PCT publications WO 90/15070 and 92/10092. These arrays are generally produced using mechanical synthesis methods or light directed synthesis methods, which incorporate a combination of photolithographic methods and solid phase oligonucleotide synthesis. (Fodor et al., *Science*, 251:767-777, (1991)). The immobilization of arrays of oligonucleotides on solid supports has been rendered possible by the development of a technology generally identified as "Very Large Scale Immobilized

Polymer Synthesis" (VLSPIS™) in which, typically, probes are immobilized in a high density array on a solid surface of a chip. Examples of VLSPIS™ technologies are provided in US Patents 5,143,854 and 5,412,087 and in PCT Publications WO 90/15070, WO 92/10092 and WO 95/11995, which describe methods for forming oligonucleotide arrays through techniques such as light-directed synthesis techniques. In designing strategies aimed at providing arrays of nucleotides immobilized on solid supports, further presentation strategies were developed to order and display the oligonucleotide arrays on the chips in an attempt to maximize hybridization patterns and diagnostic information. Examples of such presentation strategies are disclosed in PCT Publications WO 94/12305, WO 94/11530, WO 97/29212, and WO 97/31256.

[0366] A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid assays. There are several ways to produce labeled nucleic acids for hybridization or PCR including, but not limited to, oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, a nucleic acid encoding an HCV peptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides. A number of companies such as Pharmacia Biotech (Piscataway N.J.), Promega (Madison Wis.), and U.S. Biochemical Corp (Cleveland Ohio) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents, as well as, substrates, cofactors, inhibitors, magnetic particles and the like.

[0367] The presence of an HCV peptide in a protein sample obtained from a patient can also be detected by using conventional assays and the embodiments described herein. For example, antibodies that are immunoreactive with the disclosed HCV peptides can be used to screen biological samples for the presence of HCV infection. In preferred embodiments, antibodies that are reactive to the embodied HCV peptides are used to immunoprecipitate the disclosed HCV peptides from biological samples or are used to react with proteins obtained from a biological sample on Western or Immunoblots. Favored diagnostic embodiments also include enzyme-linked immunosorbant assays (ELISA),

radioimmunoassays (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies specific for the disclosed HCV peptides. Exemplary sandwich assays are described by David et al., in U.S. Patent Nos. 4,376,110 and 4,486,530. Other embodiments employ aspects of the immune-strip technology disclosed in U.S. Patent Nos. 5,290,678; 5,604,105; 5,710,008; 5,744,358; and 5,747,274.

[0368] In another preferred protein-based diagnostic, the antibodies described herein are attached to a support in an ordered array, wherein a plurality of antibodies are attached to distinct regions of the support that do not overlap with each other. As with the nucleic acid-based arrays, the protein-based arrays are ordered arrays that are designed to be "addressable" such that the distinct locations are recorded and can be accessed as part of an assay procedure. These probes are joined to a support in different known locations. The knowledge of the precise location of each probe makes these "addressable" arrays particularly useful in binding assays. For example, an addressable array can comprise a support having several regions to which are joined a plurality of antibody probes that specifically recognize HCV peptides present in a biological sample and differentiate the isotype of HCV identified herein.

[0369] By one approach, proteins are obtained from biological samples and are then labeled by conventional approaches (e.g., radioactivity, colorimetrically, or fluorescently). The labeled samples are then applied to the array under conditions that permit binding. If a protein in the sample binds to an antibody probe on the array, then a signal will be detected at a position on the support that corresponds to the location of the antibody-protein complex. Since the identity of each labeled sample is known and the region of the support on which the labeled sample was applied is known, an identification of the presence, concentration, and/or expression level can be rapidly determined. That is, by employing labeled standards of a known concentration of HCV peptide, an investigator can accurately determine the protein concentration of the particular peptide in a tested sample and can also assess the expression level of the HCV peptide. Conventional methods in densitometry can also be used to more accurately determine the concentration or expression level of the HCV peptide. These approaches are easily automated using technology known to those of skill in the art of high throughput diagnostic analysis.

[0370] In another embodiment, an approach opposite to that presented above can be employed. Proteins present in biological samples can be disposed on a support so as to create an addressable array. Preferably, the protein samples are disposed on the support at known positions that do not overlap. The presence of an HCV peptide in each sample is then determined by applying labeled antibody probes that recognize epitopes specific for the HCV peptide. Because the identity of the biological sample and its position on the array is known, an identification of the presence, concentration, and/or expression level of an HCV peptide can be rapidly determined.

[0371] That is, by employing labeled standards of a known concentration of HCV peptide, an investigator can accurately determine the concentration of peptide in a sample and from this information can assess the expression level of the peptide. Conventional methods in densitometry can also be used to more accurately determine the concentration or expression level of the HCV peptide. These approaches are also easily automated using technology known to those of skill in the art of high throughput diagnostic analysis. As detailed above, any addressable array technology known in the art can be employed. The next section describes more compositions that include the HCV nucleic acids and/or HCV peptides described herein.

Compositions comprising HCV nucleic acids or peptides

[0372] Embodiments of the invention also include NS3/4A fusion proteins or nucleic acids encoding these molecules. For instance, production and purification of recombinant protein may be facilitated by the addition of auxiliary amino acids to form a "tag". Such tags include, but are not limited to, His-6, Flag, Myc and GST. The tags may be added to the C-terminus, N-terminus, or within the NS3/4A amino acid sequence. Further embodiments include NS3/4A fusion proteins with amino or carboxy terminal truncations, or internal deletions, or with additional polypeptide sequences added to the amino or carboxy terminal ends, or added internally. Other embodiments include NS3/4A fusion proteins, or truncated or mutated versions thereof, where the residues of the NS3/4A proteolytic cleavage site have been substituted. Such substitutions include, but are not limited to, sequences where the P1' site is a Ser, Gly, or Pro, or the P1 position is an Arg, or where the P8 to P4' sequence is Ser-Ala-Asp-Leu-Glu-Val-Val-Thr-Ser-Thr-Trp-Val (**SEQ. ID. NO.: 15**).

[0373] More embodiments concern an immunogen comprising the NS3/4A fusion protein, or a truncated, mutated, or modified version thereof, capable of eliciting an enhanced immune response against NS3. The immunogen can be provided in a substantially purified form, which means that the immunogen has been rendered substantially free of other proteins, lipids, carbohydrates or other compounds with which it naturally associates.

[0374] Some embodiments contain at least one of the HCV nucleic acids or HCV peptides (e.g., **SEQ. ID. NOs.: 1-27, 35, 36 or 40-220**) joined to a support. Preferably, these supports are manufactured so as to create a multimeric agent. These multimeric agents provide the HCV peptide or nucleic acid in such a form or in such a way that a sufficient affinity to the molecule is achieved. A multimeric agent having an HCV nucleic acid or peptide can be obtained by joining the desired molecule to a macromolecular support. A "support" can be termed a carrier, a protein, a resin, a cell membrane, a capsid or portion thereof, or any macromolecular structure used to join or immobilize such molecules. Solid supports include, but are not limited to, the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, animal cells, Duracyte®, artificial cells, and others. An HCV nucleic acid or peptide can also be joined to inorganic carriers, such as silicon oxide material (e.g., silica gel, zeolite, diatomaceous earth or aminated glass) by, for example, a covalent linkage through a hydroxy, carboxy or amino group and a reactive group on the carrier.

[0375] In several multimeric agents, the macromolecular support has a hydrophobic surface that interacts with a portion of the HCV nucleic acid or peptide by a hydrophobic non-covalent interaction. In some cases, the hydrophobic surface of the support is a polymer such as plastic or any other polymer in which hydrophobic groups have been linked such as polystyrene, polyethylene or polyvinyl. Additionally, HCV nucleic acid or peptide can be covalently bound to carriers including proteins and oligo/polysaccharides (e.g. cellulose, starch, glycogen, chitosane or aminated sepharose). In these later multimeric agents, a reactive group on the molecule, such as a hydroxy or an amino group, is used to join to a reactive group on the carrier so as to create the covalent bond. Additional multimeric agents comprise a support that has other reactive groups that are chemically activated so as to attach the HCV nucleic acid or peptide. For example, cyanogen bromide activated matrices, epoxy activated matrices, thio and thiopropyl gels, nitrophenyl

chloroformate and N-hydroxy succinimide chlorformate linkages, or oxirane acrylic supports are used. (Sigma).

[0376] Carriers for use in the body, (i.e. for prophylactic or therapeutic applications) are desirably physiological, non-toxic and preferably, non-immunoresponsive. Suitable carriers for use in the body include poly-L-lysine, poly-D, L-alanine, liposomes, capsids that display the desired HCV peptide or nucleic acid, and Chromosorb[®] (Johns-Manville Products, Denver Co.). Ligand conjugated Chromosorb[®] (Synsorb-Pk) has been tested in humans for the prevention of hemolytic-uremic syndrome and was reported as not presenting adverse reactions. (*Armstrong et al. J. Infectious Diseases 171:1042-1045 (1995)*). For some embodiments, a "naked" carrier (i.e., lacking an attached HCV nucleic acid or peptide) that has the capacity to attach an HCV nucleic acid or peptide in the body of a organism is administered. By this approach, a "prodrug-type" therapy is envisioned in which the naked carrier is administered separately from the HCV nucleic acid or peptide and, once both are in the body of the organism, the carrier and the HCV nucleic acid or peptide are assembled into a multimeric complex.

[0377] The insertion of linkers, (e.g., " λ linkers" engineered to resemble the flexible regions of λ phage) of an appropriate length between the HCV nucleic acid or peptide and the support are also contemplated so as to encourage greater flexibility of the HCV peptide, hybrid, or binding partner and thereby overcome any steric hindrance that can be presented by the support. The determination of an appropriate length of linker that allows for an optimal cellular response or lack thereof, can be determined by screening the HCV nucleic acid or peptide with varying linkers in the assays detailed in the present disclosure.

[0378] A composite support comprising more than one type of HCV nucleic acid or peptide is also envisioned. A "composite support" can be a carrier, a resin, or any macromolecular structure used to attach or immobilize two or more different HCV nucleic acids or peptides. As above, the insertion of linkers, such as λ linkers, of an appropriate length between the HCV nucleic acid or peptide and the support is also contemplated so as to encourage greater flexibility in the molecule and thereby overcome any steric hindrance that can occur. The determination of an appropriate length of linker that allows for an optimal cellular response or lack thereof, can be determined by screening the HCV nucleic acid or peptide with varying linkers in the assays detailed in the present disclosure.

[0379] In other embodiments, the multimeric and composite supports discussed above can have attached multimerized HCV nucleic acids or peptides so as to create a "multimerized-multimeric support" and a "multimerized-composite support", respectively. A multimerized ligand can, for example, be obtained by coupling two or more HCV nucleic acids or peptides in tandem using conventional techniques in molecular biology. The multimerized form of the HCV nucleic acid or peptide can be advantageous for many applications because of the ability to obtain an agent with a higher affinity, for example. The incorporation of linkers or spacers, such as flexible λ linkers, between the individual domains that make-up the multimerized agent can also be advantageous for some embodiments. The insertion of λ linkers of an appropriate length between protein binding domains, for example, can encourage greater flexibility in the molecule and can overcome steric hindrance. Similarly, the insertion of linkers between the multimerized HCV nucleic acid or peptide and the support can encourage greater flexibility and limit steric hindrance presented by the support. The determination of an appropriate length of linker can be determined by screening the HCV nucleic acids or peptides in the assays detailed in this disclosure.

[0380] Embodiments also include vaccine compositions and immunogen preparations comprising the NS3/4A fusion protein, or a truncated or mutated version thereof, and, optionally, an adjuvant. The next section describes some of these compositions in greater detail.

Vaccine compositions and immunogenic preparations

[0381] Vaccine compositions and immunogenic preparations comprising, consisting of, or consisting essentially of either an embodied nucleic acid encoding a chimeric NS3/4A peptide or a chimeric NS3/4A polypeptide, or both, are contemplated. These compositions typically contain an adjuvant, but do not necessarily require an adjuvant. That is many of the nucleic acids and peptides described herein function as immunogens when administered neat. The compositions described herein (e.g., the NS3/4A chimeric immunogens and vaccine compositions containing an adjuvant, such as ribavirin) can be manufactured in accordance with conventional methods of galenic pharmacy to produce medicinal agents for administration to animals, e.g., mammals including humans. (See, e.g.,

U.S. Patent No's 6,680,059 and 6,858,590, hereby expressly incorporated by reference in their entireties).

[0382] Various nucleic acid-based vaccines are known and it is contemplated that these compositions and approaches to immunotherapy can be augmented by reformulation with ribavirin (*See, e.g.*, U.S. Pat. No. 5,589,466 and 6,235,888, hereby expressly incorporated by reference in their entireties). By one approach, for example, a gene encoding one of the NS3/4A chimeric polypeptides described herein is cloned into an expression vector capable of expressing the polypeptide when introduced into a subject. The expression construct is introduced into the subject in a mixture of adjuvant (*e.g.*, ribavirin) or in conjunction with an adjuvant (*e.g.*, ribavirin). For example, the adjuvant (*e.g.*, ribavirin) is administered shortly after the expression construct at the same site. Alternatively, RNA encoding the NS3/4A chimeric polypeptide of interest is provided to the subject in a mixture with ribavirin or in conjunction with an adjuvant (*e.g.*, ribavirin).

[0383] Where the antigen is to be DNA (*e.g.*, preparation of a DNA vaccine composition), suitable promoters include Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine and human metallothionein can be used. Examples of polyadenylation signals useful with some embodiments, especially in the production of a genetic vaccine for humans, include but are not limited to, SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal, which is in pCEP4 plasmid (Invitrogen, San Diego Calif.), referred to as the SV40 polyadenylation signal, is used.

[0384] In addition to the regulatory elements required for gene expression, other elements may also be included in a gene construct. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human actin, human myosin, human hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV. Gene constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce

multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region, which produces high copy episomal replication without integration. All forms of DNA, whether replicating or non-replicating, which do not become integrated into the genome, and which are expressible, can be used. Preferably, the genetic vaccines comprise ribavirin and a nucleic acid encoding a NS3/4A polypeptide.

[0385] More embodiments concern an immunogen comprising the chimeric NS3/4A polypeptide, or a truncated, mutated, or modified version thereof, capable of eliciting an enhanced immune response against a target antigen. The immunogen can be provided in a substantially purified form, which means that the immunogen has been rendered substantially free of other proteins, lipids, carbohydrates or other compounds with which it naturally associates.

[0386] Some embodiments contain at least one of the nucleic acids described joined to a support. Preferably, these supports are manufactured so as to create a multimeric agent. These multimeric agents provide the chimeric NS3/4A chimeric polypeptide or encoding nucleic acid in such a form or in such a way that a sufficient affinity to the molecule is achieved. A multimeric agent having a chimeric NS3/4A chimeric polypeptide or encoding nucleic acid can be obtained by joining the desired molecule to a macromolecular support. A "support" can be termed a carrier, a protein, a resin, a cell membrane, a capsid or portion thereof, or any macromolecular structure used to join or immobilize such molecules. Solid supports include, but are not limited to, the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, animal cells, DURACYTE[®], artificial cells, and others. A chimeric NS3/4A polypeptide or encoding nucleic acid can also be joined to inorganic carriers, such as silicon oxide material (e.g., silica gel, zeolite, diatomaceous earth or aminated glass) by, for example, a covalent linkage through a hydroxy, carboxy or amino group and a reactive group on the carrier.

[0387] In several multimeric agents, the macromolecular support has a hydrophobic surface that interacts with a portion of the chimeric NS3/4A chimeric polypeptide or encoding nucleic acid by a hydrophobic non-covalent interaction. In some cases, the hydrophobic surface of the support is a polymer such as plastic or any other

polymer in which hydrophobic groups have been linked such as polystyrene, polyethylene or polyvinyl. Additionally, chimeric NS3/4A polypeptides or encoding nucleic acids can be covalently bound to carriers including proteins and oligo/polysaccharides (e.g. cellulose, starch, glycogen, chitosane, aminated sepharose, or the gal epitope (e.g., gal- α -1, 3 gal- β). In these later multimeric agents, a reactive group on the molecule, such as a hydroxy or an amino group, is used to join to a reactive group on the carrier so as to create the covalent bond. Additional multimeric agents comprise a support that has other reactive groups that are chemically activated so as to attach chimeric NS3/4A polypeptides or encoding nucleic acids. For example, cyanogen bromide activated matrices, epoxy activated matrices, thio and thiopropyl gels, nitrophenyl chloroformate and N-hydroxy succinimide chloroformate linkages, or oxirane acrylic supports are used. (Sigma).

[0388] Carriers for use in the body, (i.e. for prophylactic or therapeutic applications) are desirably physiological, non-toxic and preferably, non-immunoresponsive. Suitable carriers for use in the body include poly-L-lysine, poly-D, L-alanine, liposomes, capsids that display the desired NS3/4A chimeric peptide or nucleic acid, and CHROMSORB[®] (Johns-Manville Products, Denver Co.). Ligand conjugated CHROMSORB[®] (Synsorb-Pk) has been tested in humans for the prevention of hemolytic-uremic syndrome and was reported as not presenting adverse reactions. (Armstrong et al. J. Infectious Diseases 171:1042-1045 (1995)). For some embodiments, a "naked" carrier (i.e., lacking an attached chimeric NS3/4A chimeric polypeptides or encoding nucleic acids) that has the capacity to attach a chimeric NS3/4A chimeric polypeptide or encoding nucleic acid in the body of a organism is administered. By this approach, a "prodrug-type" therapy is envisioned in which the naked carrier is administered separately from the NS3/4A chimeric polypeptide or encoding nucleic acid and, once both are in the body of the organism, the carrier and NS3/4A chimeric polypeptide or encoding nucleic acid are assembled into a multimeric complex.

[0389] The insertion of linkers of an appropriate length between the NS3/4A chimeric polypeptide or encoding nucleic acid and the support are also contemplated so as to encourage greater flexibility of the NS3/4A chimeric polypeptide, encoding nucleic acid, hybrid, or binding partner and thereby overcome any steric hindrance that can be presented by the support. The determination of an appropriate length of linker that allows for an

optimal cellular response or lack thereof, can be determined by screening the NS3/4A chimeric polypeptide or encoding nucleic acid with varying linkers in the assays detailed in the present disclosure.

[0390] A composite support comprising more than one type of NS3/4A chimeric polypeptide or encoding nucleic acid is also envisioned. A "composite support" can be a carrier, a resin, or any macromolecular structure used to attach or immobilize two or more different NS3/4A chimeric polypeptides or encoding nucleic acids. As above, the insertion of linkers, such as λ linkers, of an appropriate length between the NS3/4A chimeric polypeptide or encoding nucleic acid and the support is also contemplated so as to encourage greater flexibility in the molecule and thereby overcome any steric hindrance that can occur. The determination of an appropriate length of linker that allows for an optimal cellular response or lack thereof, can be determined by screening the NS3/4A chimeric polypeptide or encoding nucleic acid with varying linkers in the assays detailed in the present disclosure.

[0391] In other embodiments, the multimeric and composite supports discussed above can have attached multimerized NS3/4A chimeric polypeptides or encoding nucleic acids so as to create a "multimerized-multimeric support" and a "multimerized-composite support", respectively. A multimerized ligand can, for example, be obtained by coupling two or more NS3/4A chimeric polypeptides or encoding nucleic acids in tandem using conventional techniques in molecular biology. The multimerized form of NS3/4A chimeric polypeptides or encoding nucleic acids can be advantageous for many applications because of the ability to obtain an agent with a higher affinity, for example. The incorporation of linkers such as flexible λ linkers, between the individual domains that make-up the multimerized agent can also be advantageous for some embodiments. The insertion of λ linkers of an appropriate length between protein binding domains, for example, can encourage greater flexibility in the molecule and can overcome steric hindrance. Similarly, the insertion of linkers between the multimerized NS3/4A chimeric polypeptides or encoding nucleic acids and the support can encourage greater flexibility and limit steric hindrance presented by the support. The determination of an appropriate length of linker can be determined by screening the NS3/4A chimeric polypeptides or encoding nucleic acid in the assays detailed in this disclosure.

[0392] Aspects of the present invention also relate to a modified hepatitis C virus (HCV) NS3/4A protein that is linked to a pendent hapten (e.g., a T cell epitope) through chemically-reactive amino acid residue, which may be, optionally, inserted into or attached to (e.g., a linker on either the N terminal or C terminal end) the NS3/4A sequence. Such an introduced or already present chemically-reactive amino acid residue is characterized in that it has a chemically-reactive side chain that provides a chemical group for pendently linking the NS3/4A polypeptide to the hapten. Typically, the chemically-reactive amino acid residue is a lysine, cysteine, or histidine residue or a carboxyl-containing residue such as aspartic acid or glutamic acid, preferably lysine or a carboxyl-containing residue, and most preferably lysine. The hapten bonded to the chemically-reactive amino acid residue is any compound of interest for generating an immune response, and is typically a T cell epitope. Preferably, the hapten is a polypeptide hapten, a carbohydrate hapten, or a non-peptidal/non-saccharidal (chemical) hapten. In some embodiments, the hapten is a pathogen-related hapten, for example, a T cell epitope provided herein. The word "hapten" is used in this context to describe molecules that are capable of stimulating an immune response (e.g., production of antibody) when chemically coupled to NS3/4A. The word is often used for small nonantigenic molecules in the art, but herein, it merely refers to the molecule that is to be pendently linked to NS3/4A, even if it is antigenic or not small. The chemically-reactive amino acid residue can be at any position within the epitope and NS3/4A polypeptide or attached to either end of these molecules (N terminal or C terminal). As stated above, the introduced chemically-reactive amino acid residue has a chemically-reactive side chain that provides a functional group for derivatizing the NS3/4A polypeptide (e.g., conjugating a T cell epitope to the NS3/4A). Useful side chain functional groups include epsilon-amino groups, beta-or gamma-carboxyl groups, thiol (-SH) groups and aromatic rings (e.g. tyrosine and histidine). The chemically-reactive amino acid residue is typically a lysine, cysteine, or histidine residue or a carboxyl-containing residue such as aspartic acid or glutamic acid. Lysine is a particularly preferred chemically-reactive amino acid residue. In addition of the use of an individual chemically-reactive amino acid residue in the insert such as aspartic acid or lysine, substantially any sequence of the desired length that contains a chemically-reactive amino acid residue can be used.

[0393] Any hapten against which antibody production is desired can be linked to an NS3/4A peptide or fragment thereof, as described herein, to form an immunogenic composition. The hapten of interest typically is T cell epitope. The hapten can be a polypeptide, a carbohydrate (saccharide), or a non-polypeptide, non-carbohydrate chemical such as 2,4-dinitrobenzene, however.

[0394] Methods for operatively linking individual haptens to a protein or polypeptide through an amino acid residue side chain of the protein or polypeptide to form a pendently-linked immunogenic conjugate, e.g., a branched-chain polypeptide polymer, are well known in the art. Those methods include linking through one or more types of functional groups on various side chains and result in the carrier protein polypeptide backbone being pendently linked--covalently linked (coupled) to the hapten but separated by at least one side chain.

[0395] Methods for linking carrier proteins to haptens using each of the above functional groups are described in Erlanger, *Method of Enzymology*, 70:85 (1980), Aurameas, et al., *Scand. J. Immunol.*, Vol. 8, Suppl. 7, 7-23 (1978) and U.S. Pat. No. 4,493,795 to Nestor et al., all of which are hereby expressly incorporated by reference in their entireties. In addition, a site-directed coupling reaction, as described in Rodwell et al., *Biotech.*, 3, 889-894 (1985), herein expressly incorporated by reference in its entirety, can be carried out so that the biological activity of the polypeptides is not substantially diminished.

[0396] Furthermore, as is well known in the art, both the NS3/4A protein or fragment thereof and a polypeptide hapten can be used in their native form or their functional group content can be modified by succinylation of lysine residues or reaction with cysteine-thiolactone. A sulfhydryl group can also be incorporated into either molecule by reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(3-dithiopyridyl)propionate.

[0397] The NS3/4A peptide or fragment thereof or hapten can also be modified to incorporate a spacer arm, such as hexamethylene diamine or other bifunctional molecules of similar size, to facilitate the pendent linking.

[0398] Methods for covalent bonding of a polypeptide hapten are extremely varied and are well known by workers skilled in the immunological arts. For example, following U.S. Pat. No. 4,818,527, m-maleimidobenzoyl-N-hydroxysuccinimide ester (ICN

Biochemicals, Inc.) or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce), all of which are hereby expressly incorporated by reference in their entirety, is reacted with a NS3/4A protein or fragment thereof to form an activated molecule. That activated carrier is then reacted with a polypeptide that either contains a terminal cysteine or to which an additional amino- or carboxy-terminal cysteine residue has been added to form a covalently bonded NS3/4A conjugate. As an alternative example, the amino group of a polypeptide hapten can be first reacted with N-succinimidyl 3-(2-pyridylthio)propionate (SPDP, Pharmacia), and that thiol-containing polypeptide can be reacted with the activated NS3/4A after reduction. Of course, the sulfur-containing moiety and double bond-containing Michael acceptor can be reversed. These reactions are described in the supplier's literature, and also in Kitagawa, et al., *J. Biochem.*, 79:233 (1976) and in Lachmann et al., in 1986 *Synthetic Peptides as Antigens*, (Ciba Foundation Symposium 119), pp. 25-40 (Wiley, Chichester: 1986), all of which are hereby expressly incorporated by reference in their entirety.

[0399] U.S. Pat. No. 4,767,842, herein expressly incorporated by reference in its entirety, teaches several modes of covalent attachment between a carrier and polypeptide that are useful here. In one method, tolylene diisocyanate is reacted with the NS3/4A or a fragment thereof in a dioxane-buffer solvent at zero degrees C to form an activated molecule. A polypeptide hapten (e.g., a T cell epitope) is thereafter admixed and reacted with the activated NS3/4A to form the covalently bonded NS3/4A conjugate.

[0400] Particularly useful are a large number of heterobifunctional agents that form a disulfide link at one functional group end and a peptide link at the other, including N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). This reagent creates a disulfide linkage between itself and a thiol in either the NS3/4A or fragment thereof or the hapten, for example a cysteine residue in a polypeptide hapten, and an amide linkage on the coupling partner, for example the amino on a lysine or other free amino group in the NS3/4A. A variety of such disulfide/amide forming agents are known. (See for example *Immun. Rev.* (1982) 62:185, herein expressly incorporated by reference in its entirety). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl)cyclohexane-1-carboxylic acid

and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. The particularly preferred coupling agent is succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) obtained from Pierce Company, Rockford, Ill. The foregoing list is not meant to be exhaustive, and modifications of the named compounds can clearly be used.

[0401] A polypeptide hapten (e.g., a T cell epitope) can be obtained in a number of ways well known in the art. Usual peptide synthesis techniques can be readily utilized. For example, recombinant and PCR-based techniques to produce longer peptides are useful. Because the desired sequences are usually relatively short, solid phase chemical synthesis is useful.

[0402] As discussed below, DNA sequences that encode a variety of polypeptide haptens (e.g., T cell epitopes) are known in the art. The coding sequence for peptides of the length contemplated herein can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981). Of course, by chemically synthesizing the coding sequence, any desired modification can be made simply by substituting the appropriate bases for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors now commonly available in the art, and the regulating vectors used to transform suitable hosts to produce the desired protein.

[0403] A number of such vectors and suitable host systems are now available. For example promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. Typical of such vector plasmids are, for example, pUC8, and pUC13 available from J. Messing, at the University of Minnesota (see, e.g., Messing et al., Nucleic Acids Res. 9:309 (1981)) or pBR322, available from New England Biolabs. Suitable promoters include, for example, the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang. et al., Nature 198:1056 (1977) and the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8:4057 (1980)). The resulting expression vectors are transformed into suitable bacterial hosts using the calcium chloride method described by Cohen, et al., Proc. Natl. Acad. Sci. U.S.A. 69:2110 (1972). Successful transformants may produce the desired polypeptide fragments at higher levels than those found in strains normally producing the intact pili. Of

course, yeast or mammalian cell hosts can also be used, employing suitable vectors and control sequences.

[0404] Embodiments also include methods of using vaccine compositions and immunogen preparations comprising the NS3/4A chimeric polypeptides or encoding nucleic acids, or a truncated or mutated version thereof, and, optionally, an adjuvant. The next section describes some of these compositions in greater detail.

Methods of using the vaccine compositions and immunogen preparations

[0405] Routes of administration of the embodiments described herein include, but are not limited to, transdermal, parenteral, gastrointestinal, transbronchial, and transalveolar. Transdermal administration can be accomplished by application of a cream, rinse, gel, etc. capable of allowing the compositions described herein to penetrate the skin. Parenteral routes of administration include, but are not limited to, electrical or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. Transbronchial and transalveolar routes of administration include, but are not limited to, inhalation, either via the mouth or intranasally.

[0406] Compositions that are suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams, and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device ("transdermal patch"). Examples of suitable creams, ointments, etc. can be found, for instance, in the Physician's Desk Reference. Examples of suitable transdermal devices are described, for instance, in U.S. Patent No. 4,818,540 issued April 4, 1989 to Chinen, et al., hereby expressly incorporated by reference in its entirety.

[0407] Compositions that are suitable for parenteral administration include, but are not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline, phosphate buffered saline and oil preparations for injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection.

[0408] Compositions that are suitable for transbronchial and transalveolar administration include, but not limited to, various types of aerosols for inhalation. Devices

suitable for transbronchial and transalveolar administration of these are also embodiments. Such devices include, but are not limited to, atomizers and vaporizers. Many forms of currently available atomizers and vaporizers can be readily adapted to deliver vaccines having ribavirin and an antigen.

[0409] Compositions that are suitable for gastrointestinal administration include, but not limited to, pharmaceutically acceptable powders, pills or liquids for ingestion and suppositories for rectal administration.

[0410] The nucleic acid constructs described herein, in particular, may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Alternatively, the genetic vaccine may be introduced by various means into cells that are removed from the individual. Such means include, for example, ex vivo transfection, electroporation, microinjection and microprojectile bombardment. After the gene construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have gene constructs incorporated therein can be implanted into the individual even if the vaccinated cells were originally taken from another individual.

[0411] According to some embodiments, the gene construct is administered to an individual using a needleless injection device. In other embodiments, the gene construct comprising the antigen of interest is provided to an individual in need of an immune response to said antigen using an electroporation device (e.g., a needle device or a needleless device). According to some embodiments, the gene construct is simultaneously administered to an individual intradermally, subcutaneously and intramuscularly using a needleless injection device. Needleless injection devices, multi-needle electroporation devices, and nucleic acid electroporation devices, in general, are well known and widely available (*See e.g.*, US 5,273,525, EP 1240917 B1, US 5,702,359, EP 0874663B1, US 6,418,341, US 6,763,264 US 6,055,453, US 6,233,482, US 6,068,650, US 6,014,584, US 6,241,701, US 6,516,223, US 6,678,556, and US 6,110,161, hereby expressly incorporated by reference in their entireties).

[0412] One having ordinary skill in the art can, following the teachings herein, use needleless or needled electroporation devices (e.g., providing the nucleic acid construct by hypodermic needle followed by electroporation at the injection site) to deliver genetic material to cells of an individual. These gene construct delivery devices are well suited to

deliver genetic material to all tissue. They are particularly useful to deliver genetic material to skin and muscle cells. In some embodiments, a needleless injection device may be used to propel a liquid or dissolvable substrate or carrier that comprises the nucleic acid construct (e.g., ballistic transformation) toward the surface of the individual's skin. The liquid is propelled at a sufficient velocity such that upon impact with the skin the liquid penetrates the surface of the skin, permeates the skin and muscle tissue therebeneath. Thus, the genetic material is simultaneously administered intradermally, subcutaneously and intramuscularly. In some embodiments, a needleless injection device may be used to deliver genetic material to tissue of other organs in order to introduce a nucleic acid molecule to cells of that organ.

[0413] Preferred embodiments include methods of enhancing an immune response to a desired antigen by providing an animal in need with an amount of adjuvant (e.g., ribavirin) and one or more of the nucleic acid or polypeptide compositions disclosed herein that is effective to enhance said immune response. In these embodiments, an animal in need of an enhanced immune response to an antigen/target is identified by using currently available diagnostic testing or clinical evaluation. By one approach, for example, an individual infected with a virus, or afflicted with cancer, is provided with the vaccine compositions described above in an amount sufficient to elicit a cellular and humoral immune response to a viral or cancer TCE so as to protect said individual from becoming infected with the virus, or to treat the cancer from which the TCE is derived. In another embodiment, an individual infected with a virus is identified and provided with a vaccine composition comprising ribavirin and either a nucleic acid or polypeptide composition described herein, that includes a TCE from the virus and NS3/4A sequences in an amount sufficient to enhance the cellular and humoral immune response against the viral TCE so as to reduce or eliminate the viral infection.

[0414] The following Example describes the systematic mutation of residues in the NS3 protease domain in order to elucidate the potential insertion sites for TCEs or TCEs and linkers, in which the chimeric NS3/4A proteins retain protease activity.

EXAMPLE 14

[0415] The serine protease cleavage domain of NS3/NS4A resides in the first 181 amino acids of the peptide (Lin, C. *et al.*, *J. Virol.*, 68(12):8147-8157 (1994)). Figure 17

depicts the amino acid sequence of the protease cleavage domain of the NS3/NS4A polypeptide from HCV isolate disclosed herein (**SEQ ID NO: 39**). To identify amino acid residues that affect protease activity in the HCV isolate, mutations were made in each of the 181 amino acids of the protease cleavage domain. Briefly, the NS3/NS4A-pVAX plasmid (described in Example 1) was used as a template for site-directed mutagenesis using the QUICKCHANGE™ mutagenesis kit (Stratagene), following the manufacturer's recommendations. Using this approach, NS3/NS4A-pVAX constructs encoding the polypeptides of (**SEQ ID NO's: 40 through 220 and 1329-1339**) were made. These constructs encode NS3/NS4A polypeptides in which every residue other than alanine in (**SEQ ID NO: 39**) is changed to an alanine, and where every alanine in (**SEQ ID NO: 39**) is changed to a glycine. The resulting plasmids were sequenced to verify that the NS3/NS4A-pVAX vectors had been correctly made. Plasmids were grown in competent BL21 *E. coli*, and subsequently purified using Qiagen DNA purification columns (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Purified plasmid DNA was dissolved in phosphate buffered saline (PBS).

[0416] The resulting plasmids were transcribed and translated *in vitro*, and the resulting polypeptides were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *In vitro* transcription and translation were performed using the T7 coupled reticulocyte lysate TNT™ system (Promega, Madison, WI) according to the manufacturer's instructions. All *in vitro* translation reactions of the constructs were carried out at 30°C with ³⁵S-labeled methionine (Amersham International, Plc, Buckinghamshire, UK). The labeled proteins were separated by 12% SDS-PAGE and visualized by exposure to X-ray film (Hyper Film-MP, Amersham) for 6-18 hours.

[0417] When the assay described above is performed with wtNS3/NS4A-pVAX, the protease activity of wtNS3/NS4A protein (**SEQ ID NO: 2**) is such that two protein bands are visualized on the autorad of the gel: a protein band of approximately 67kDa, which is consistent with the size of the NS3/NS4A uncleaved protein, and a protein band of approximately 61kDa, which corresponds to the NS3 cleavage product from the reaction. Each of the 181 mutant NS3/NS4A-pVAX constructs was tested in the assay described above. For each mutant construct assayed, the amount of uncleaved (67kDa) versus cleaved NS3/NS4A (61kDa cleavage product) was compared between the wtNS3/NS4A construct

and the NS3/NS4A mutant construct, as a measure of how each mutation affected the protease activity. As shown in **Table 21**, the following NS3/NS4A constructs have amino acid substitutions that completely abolished protease activity: **SEQ ID NOs: 87, 92, 96, 120, 124, 130, 136, 138, 162, 163, 178, 179, 184, 192, 208, and 214**. In reference to NS3 protease activity, the term “completely abolished” is meant to refer to polypeptides that have less than, equal to, or any number in between about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% and 1% of the NS3 protease activity compared to the protease activity of a wild type NS3 polypeptide or NS3/4A polypeptide (*e.g.*, **SEQ ID NO:36**). In reference to NS3 protease activity, the term “reduced” is meant to refer to polypeptides that have less than, equal to, or any number in between about 100%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15% and 10% of the NS3 protease activity compared to the protease activity of a wild type NS3 polypeptide or NS3/4A polypeptide (*e.g.*, **SEQ ID NO:36**). The following eight constructs have mutations that result in reduced protease activity: **SEQ ID NOs: 83, 133, 145, 147, 165, 182, 183, and 188**.

[0418] As shown in **Table 22**, twenty two constructs have substitutions that result in enhanced (**SEQ ID NOs: 45, 50, 52, 53, 69, 98, 103, 112, 115, 125, 150, 161, 173, 175, 180, 200, 205, and 216**), or greatly enhanced protease activity (**SEQ ID NOs: 91, 97, and 197**). In reference to NS3 protease activity, the term “enhanced” and “greatly enhanced” is meant to refer to polypeptides that have greater than, equal to, or any number in between about 100%, 101%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, 150%, 155%, 160%, 165%, 170%, 175%, 180%, 185%, 190%, 195%, 200%, 210%, 220%, 230%, 240%, 250%, 260%, 270%, 280%, 290%, 300%, 310%, 320%, 330%, 340%, 350%, 360%, 370%, 380%, 390%, 400%, 425%, 450%, 475%, 500%, 600% and 700% of the NS3 protease activity compared to the protease activity of a wild type NS3 polypeptide or NS3/4A polypeptide (*e.g.*, **SEQ ID NO:36**).

Table 21

Leu44Ala (SEQ ID NO:83)	Reduced
Ile48Ala (SEQ ID NO: 87)	Abolished
Trp53Ala (SEQ ID NO: 92)	Abolished
His57Ala (SEQ ID NO: 96)	Abolished
Asp81Ala (SEQ ID NO: 120)	Abolished

Trp85Ala (SEQ ID NO: 124)	Abolished
Ala91Gly (SEQ ID NO: 130)	Abolished
Leu94Ala (SEQ ID NO: 133)	Reduced
Cys97Ala (SEQ ID NO: 136)	Abolished
Cys99Ala (SEQ ID NO: 138)	Abolished
Leu106Ala (SEQ ID NO: 145)	Reduced
Thr108Ala (SEQ ID NO: 147)	Reduced
Arg 123Ala (SEQ ID NO: 162)	Abolished
Gly124Ala (SEQ ID NO: 163)	Abolished
Leu126Ala (SEQ ID NO: 165)	Reduced
Ser139Ala (SEQ ID NO: 178)	Abolished
Gly140Ala (SEQ ID NO: 179)	Abolished
Leu143Ala (SEQ ID NO: 182)	Reduced
Leu144Ala (SEQ ID NO: 183)	Reduced
Cys145Ala (SEQ ID NO: 184)	Abolished
His149Ala (SEQ ID NO: 188)	Reduced
Ile153Ala (SEQ ID NO: 192)	Abolished
Phe169Ala (SEQ ID NO: 208)	Abolished
Leu175Ala (SEQ ID NO: 214)	Abolished

Table 22

Mutation	Activity
Tyr6Ala (SEQ ID NO: 45)	Enhanced
Arg11Ala (SEQ ID NO: 50)	Enhanced
Leu13Ala (SEQ ID NO: 52)	Enhanced
Leu14Ala (SED ID NO: 53)	Enhanced
Glu30Ala (SEQ ID NO: 69)	Enhanced
Cys52Ala (SEQ ID NO: 91)	Greatly enhanced
Gly58Ala (SEQ ID NO: 97)	Greatly enhanced
Ala59Gly (SEQ ID NO: 98)	Enhanced

Ile64Ala (SEQ ID NO: 103)	Enhanced
Gln73Ala (SEQ ID NO: 112)	Enhanced
Thr76Ala (SEQ ID NO: 115)	Enhanced
Pro86Ala (SEQ ID NO: 125)	Enhanced
Ala111Gly (SEQ ID NO: 150)	Enhanced
Gly 122Ala (SEQ ID NO: 161)	Enhanced
Tyr 134Ala (SEQ ID NO: 173)	Enhanced
Lys 136Ala (SEQ ID NO: 175)	Enhanced
Gly 141Ala (SEQ ID NO: 180)	Enhanced
Val158Ala (SEQ ID NO: 197)	Greatly Enhanced
Arg161Ala (SEQ ID NO: 200)	Enhanced
Ala166Gly (SEQ ID NO: 205)	Enhanced
Thr177Ala (SEQ ID NO: 216)	Enhanced

[0419] Protease activity is associated with viral assembly and maturation (See, e.g., Babé *et al.*, *Cell*, 91:427-430 (1997)). Accordingly, mutant NS3/NS4A polypeptides with altered protease activity and their encoding nucleic acids are useful in the immunogenic compositions described herein. The fragments listed in **TABLES 21-22** are preferred immunogens that can be incorporated with or without an adjuvant (e.g., ribavirin) into a composition for administration to an animal so as to induce an immune response in said animal to HCV.

[0420] As shown in **TABLE 23**, the following NS3/4A constructs have amino acid substitutions that did not have a large effect (SEQ ID NOs: 40, 48-49, 54, 56, 60-61, 66, 72, 74-75, 77-79, 82, 85, 89, 100-102, 107-110, 113-114, 121, 131, 144, 146, 148-149, 152-153, 156, 160, 166, 167, 170-171, 177, 181, 185-186, 189-190, 194-195, 198-199, 204, 206, 209, 210, 213, 215, and 217), or did not have any detectable effect on protease activity (SEQ ID NOs: 41-44, 46, 47, 51, 55, 58, 59, 62, 65, 67-68, 70-71, 73, 76, 80-81, 84, 86, 88, 90, 93,

95, 99, 104-106, 111, 116-119, 123, 127-129, 134-135, 137, 139-143, 151, 154-155, 157-159, 164, 168-169, 172, 176, 191, 193, 196, 201-203, 211-212, and 218-220). The fragments listed in **TABLE 22** are preferred immunogens that can be incorporated with or without an adjuvant (e.g., ribavirin) into a composition for administration to an animal so as to induce an immune response in said animal to HCV.

Table 23

Mutation	Activity
Ala1Gly (SEQ ID NO: 40)	Little Effect
Pro2Ala (SEQ ID NO: 41)	No Effect
Ile3Ala (SEQ ID NO: 42)	No Effect
Thr4Ala (SEQ ID NO: 43)	No Effect
Ala5Gly (SEQ ID NO: 44)	No Effect
Ala7Gly (SEQ ID NO: 46)	No Effect
Gln8Ala (SEQ ID NO: 47)	No Effect
Gln9Ala (SEQ ID NO: 48)	Little Effect
Thr10Ala (SEQ ID NO: 49)	Little Effect
Gly12Ala (SEQ ID NO: 51)	No Effect
Gly15Ala (SEQ ID NO: 54)	Little Effect
Cys16Ala (SEQ ID NO: 55)	No Effect
Ile17Ala (SEQ ID NO: 56)	Little Effect
Thr19Ala (SEQ ID NO: 58)	No Effect
Ser20Ala (SEQ ID NO: 59)	No Effect
Leu21Ala (SEQ ID NO: 60)	Little Effect
Thr22Ala (SEQ ID NO: 61)	Little Effect
Gly23Ala (SEQ ID NO: 62)	No Effect
Lys26Ala (SEQ ID NO: 65)	No Effect
Asn27Ala (SEQ ID NO: 66)	Little Effect
Gln28Ala (SEQ ID NO: 67)	No Effect
Val29Ala (SEQ ID NO: 68)	No Effect
Gly31Ala (SEQ ID NO: 70)	No Effect
Glu32Ala (SEQ ID NO: 71)	No Effect

Mutation	Activity
Val33Gly (SEQ ID NO: 72)	Little Effect
Gln34Ala (SEQ ID NO: 73)	No Effect
Ile35Ala (SEQ ID NO: 74)	Little Effect
Val36Ala (SEQ ID NO: 75)	No Effect
Ser37Ala (SEQ ID NO: 76)	Little Effect
Thr38Ala (SEQ ID NO: 77)	Little Effect
Ala39Gly (SEQ ID NO: 78)	Little Effect
Ala40Gly (SEQ ID NO: 79)	Little Effect
Gln41Ala (SEQ ID NO: 80)	No Effect
Thr42Ala (SEQ ID NO: 81)	No Effect
Phe43Ala (SEQ ID NO: 82)	Little Effect
Ala45Gly (SEQ ID NO: 84)	No Effect
Thr46Ala (SEQ ID NO: 85)	Little Effect
Cys47Ala (SEQ ID NO: 86)	No Effect
Gln49Ala (SEQ ID NO: 88)	No Effect
Gly50Ala (SEQ ID NO: 89)	Little Effect
Val51Ala (SEQ ID NO: 90)	Little Effect
Thr54Ala (SEQ ID NO: 93)	No Effect
Arg161Ala (SEQ ID NO: 95)	No Effect
Ala56Gly (SEQ ID NO: 99)	No Effect
Phe57Ala (SEQ ID NO: 100)	Little Effect
Leu58Ala (SEQ ID NO: 101)	No Effect
Thr63Ala (SEQ ID NO: 102)	Little Effect
Thr64Ala (SEQ ID NO: 103)	No Effect
Ala65Gly (SEQ ID NO: 104)	No Effect
Ser66Ala (SEQ ID NO: 105)	No Effect
Pro67Ala (SEQ ID NO: 106)	No Effect
Lys68Ala (SEQ ID NO: 107)	Little Effect
Gly69Ala (SEQ ID NO: 108)	Little Effect
Pro70Ala (SEQ ID NO: 109)	Little Effect

Mutation	Activity
Val71Ala(SEQ ID NO: 110)	Little Effect
Ile72Ala(SEQ ID NO: 111)	Little Effect
Met74Ala(SEQ ID NO: 113)	Little Effect
Tyr75Ala(SEQ ID NO: 114)	Little Effect
Gln77Ala(SEQ ID NO: 116)	No Effect
Val78Ala(SEQ ID NO: 117)	No Effect
Asp79Ala(SEQ ID NO: 118)	No Effect
Gln80Ala(SEQ ID NO: 119)	No Effect
Leu82Ala(SEQ ID NO: 121)	Little Effect
Gly84Ala(SEQ ID NO: 123)	No Effect
Pro88Ala(SEQ ID NO: 127)	No Effect
Gln89Ala(SEQ ID NO: 128)	No Effect
Gly90Ala(SEQ ID NO: 129)	No Effect
Arg92Ala(SEQ ID NO: 131)	Little Effect
Thr95Ala(SEQ ID NO: 134)	No Effect
Pro96Ala(SEQ ID NO: 135)	No Effect
Thr98Ala(SEQ ID NO: 137)	No Effect
Gly100Ala(SEQ ID NO: 139)	No Effect
Ser101Ala(SEQ ID NO: 140)	No Effect
Ser102Ala(SEQ ID NO: 141)	No Effect
Asp103Ala(SEQ ID NO: 142)	No Effect
Leu104Ala(SEQ ID NO: 143)	No Effect
Try105Ala(SEQ ID NO: 144)	Little Effect
Val107Ala(SEQ ID NO: 146)	Little Effect
Arg109Ala(SEQ ID NO: 148)	Little Effect
His110Ala(SEQ ID NO: 149)	Little Effect
Asp112Ala(SEQ ID NO: 151)	No Effect
Val113Ala(SEQ ID NO: 152)	Little Effect
Ile114Ala(SEQ ID NO: 153)	Little Effect
Pro115Ala(SEQ ID NO: 154)	No Effect

Mutation	Activity
Val116Ala(SEQ ID NO: 155)	No Effect
Arg118Ala(SEQ ID NO: 157)	No Effect
Arg119Ala(SEQ ID NO: 158)	Little Effect
Gly120Ala(SEQ ID NO: 159)	No Effect
Asp121Ala(SEQ ID NO: 160)	Little Effect
Ser125Ala(SEQ ID NO: 164)	No Effect
Leu127Ala(SEQ ID NO: 166)	Little Effect
Ser128Ala(SEQ ID NO: 167)	Little Effect
Pro129Ala(SEQ ID NO: 168)	No Effect
Arg130Ala(SEQ ID NO: 169)	No Effect
Pro131Ala(SEQ ID NO: 170)	Little Effect
Ile132Ala(SEQ ID NO: 171)	Little Effect
Ser133Ala(SEQ ID NO: 172)	No Effect
Gly137Ala(SEQ ID NO: 176)	No Effect
Ser138Ala(SEQ ID NO: 177)	Little Effect
Pro142Ala(SEQ ID NO: 181)	Little Effect
Pro146Ala(SEQ ID NO: 185)	Little Effect
Ala147Gly(SEQ ID NO: 186)	Little Effect
Ala150Gly(SEQ ID NO: 189)	Little Effect
Val151Gly(SEQ ID NO: 190)	Little Effect
Gly152Ala(SEQ ID NO: 191)	No Effect
Phe154Ala(SEQ ID NO: 193)	No Effect
Arg155Ala(SEQ ID NO: 194)	Little Effect
Ala156Gly(SEQ ID NO: 195)	Little Effect
Ala157Gly(SEQ ID NO: 196)	No Effect
Cys159Ala(SEQ ID NO: 198)	Little Effect
Thr160Ala(SEQ ID NO: 199)	Little Effect
Gly162Ala(SEQ ID NO: 201)	No Effect
Val163Ala(SEQ ID NO: 202)	No Effect
Ala164Gly(SEQ ID NO: 203)	No Effect

Mutation	Activity
Lys165Ala(SEQ ID NO: 204)	Little Effect
Val167Ala(SEQ ID NO: 206)	Little Effect
Ile170Ala(SEQ ID NO: 209)	Little Effect
Pro171Ala(SEQ ID NO: 210)	Little Effect
Val172Ala(SEQ ID NO: 211)	No Effect
Glu173Ala(SEQ ID NO: 212)	No Effect
Ser174Ala(SEQ ID NO: 213)	Little Effect
Glu176Ala(SEQ ID NO: 215)	Little Effect
Thr178Ala(SEQ ID NO: 217)	Little Effect
Met179Ala(SEQ ID NO: 218)	No Effect
Arg180Ala(SEQ ID NO: 219)	No Effect
Ser181Ala(SEQ ID NO: 220)	No Effect

[0421] The mutant HCV genes and the encoded polypeptides disclosed herein are useful as novel research tools for drug discovery. Specifically, polypeptides exhibiting enhanced protease activity can be used in assays to identify novel compounds that inhibit protease activity. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

[0422] The HCV genes encoding polypeptides with altered protease activity are useful in the creation of transgenic organisms, as described herein in paragraphs [0183]-[0184]. Transgenic organisms expressing mutant HCV polypeptides are useful as model organisms for the study of HCV replication and life cycle.

EXAMPLE 15

[0423] Two particular mutants, Val1055Ala (corresponding to Val29Ala of **SEQ ID NO: 68**) and Gln1060Ala (corresponding to Gln34Ala of **SEQ ID NO: 73**), were tested

for their ability to proteolytically cleave the NS3-NS4A junction while not cleaving the human IPS-1 molecule to Δ IPS-1. As detailed in above, Val29Ala of **SEQ ID NO: 68** and Gln34Ala of **SEQ ID NO: 73** were tested for their ability to affect the protease activity in the HCV isolate. These mutants were found through mutagenesis of the NS3 protease domain wherein the residues depicted in Figure 18 were replaced with Alanine or Glycine and tested for their effect on cleavage of the NS3-NS4A cleavage site (also depicted in Figure 18). Also mentioned above, these two mutants did not have a large effect on protease activity in the HCV isolate. These mutants were further tested for their ability to cleave the human IPS-1 molecule to Δ IPS-1.

[0424] Cells were cotransfected with a plasmid coding for the human IPS-1 gene as well as a pVAX1 plasmid expressing the mutant NS3/4A gene. 36 hours later the cells were lyse. Cleaved and uncleaved IPS-1 peptides were visualized on an SDS-PAGE gel after a Western blot with antibodies specific for the IPS-1 fragments. As shown in Figures 19A and 19B Val1055Ala (corresponding to Val29Ala of **SEQ ID NO: 68**) and Gln1060Ala (corresponding to Gln34Ala of **SEQ ID NO: 73**) were not able to cleave IPS-1 to Δ IPS-1.

EXAMPLE 16

[0425] A particular mutant, containing two amino acid mutations Val1055Ala and Gln1060Ala (corresponding to Val29Ala and Gln34Ala, respectively, of **SEQ ID NO: 1329**), is created by combining the mutations present in **SEQ ID NOs.: 68 and 73**. The residues referred to as 1055 and 1060 vary by 1026 amino acids from the listed sequences as the NS3/4A gene begins at residue 1026 of the HCV polyprotein. This mutant is tested for its affect on the proteolytic cleavage of the NS3-NS4A junction as in Example 15. Results show that the mutant does not have a large effect on protease activity in the HCV isolate.

[0426] This mutant is further tested for its ability to cleave the human IPS-1 molecule to Δ IPS-1 as detailed above. Results show that the IPS-1 gene is not cleaved to Δ IPS-1 by the mutant represented by **SEQ ID NO: 1329**.

EXAMPLE 17

[0427] NS3 protease mutants represented by **SEQ ID NOs: 1330-1339** are tested for their ability to affect NS3 protease cleavage at the NS3-NS4A protease cleavage site, as

explained in above. Mutants having no effect, little effect, no substantial effect, or heightened effect on protease cleavage at the NS3-NS4A protease cleavage site are selected for further testing.

[0428] These mutants are further tested for their ability to cleave the human IPS-1 molecule to Δ IPS-1 as detailed above. Mutants that cannot cleave IPS-1 to Δ IPS-1 are selected as favorable mutants.

EXAMPLE 18

[0429] Mutants containing two or more amino acid mutations, created by combining any number of favorable mutants described above, are created. These mutants are tested for their ability to affect NS3 protease cleavage at the NS3-NS4A protease cleavage site, as detailed in above. Mutants having no effect, little effect, no substantial effect, or heightened effect on protease cleavage at the NS3-NS4A protease cleavage site are selected for further testing.

[0430] These mutants are further tested for their ability to cleave the human IPS-1 molecule to Δ IPS-1 as detailed above. Mutants that cannot cleave IPS-1 to Δ IPS-1 are selected as favorable mutants.

[0431] Although the invention has been described with reference to embodiments and examples, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

[0432] **[0273]** The next example demonstrates that chimeric NS3/4A nucleic acids and encoded polypeptides described herein prime CTL responses to the T cell epitopes encoded therein.

EXAMPLE 19

[0433] The Hepatitis B viral core protein (HBc) has been disclosed as an immunogenic moiety that stimulates the T cell response of an immunized host animal. See, e.g., U.S. Pat. No. 4,818,527, U.S. Pat. No. 4,882,145 and U.S. Pat. No. 5,143,726. More particularly, the sequence of **SEQ ID NO:1014** of the Hepatitis B core protein has been shown to elicit a specific T-cell response when administered to mice. To assess the ability of

SEQ ID NO:1014 DNA constructs to prime CTLs, the nucleic acid of **SEQ ID NO:1015** is cloned into the pVAX1 expression vector (Invitrogen, Carlsbad, CA) to create HBcAg-pVAX1.

[0434] Plasmids are grown in BL21 *E. coli* cells, and sequenced for accuracy. Plasmid DNA used for *in vivo* vaccination is purified using Qiagen DNA purification columns, according to the manufacturer's instructions (Qiagen GmbH, Hilden, FRG). The concentration of the resulting plasmid DNA is determined spectrophotometrically (Dynaquant, Pharmacia Biotech, Uppsala, Sweden) and the purified DNA is dissolved in sterile phosphate buffered saline (PBS) at a concentration of 1 mg/ml.

[0435] Groups of eight to ten C57/BL6 mice are primed with HBcAg-pVAX1 intra muscularly (*i.m.*) or using a gene gun. For *i.m.* delivery, mice are immunized by needle injections of 100 µg plasmid DNA given intramuscularly to the tibialis anterior (TA) muscle. 5 days prior to DNA immunization, mice are injected intramuscularly with 50 µl per TA muscle of 0.01 mM cardiotoxin (Latoxan) in 0/9% sterile saline. The mice are boosted with a second injection of 100 µg plasmid DNA four weeks subsequent to the first DNA immunization. For gene gun delivery, plasmid DNA is linked to gold particles according to protocols supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Prior to immunization, the injection area is shaved and the immunization is performed according to the manufacturer's protocol. Each injection dose contains 4 µg of plasmid DNA. Immunizations are performed on weeks 0 and 4.

[0436] The presence of CTLs specific for **SEQ ID NO:1014** is assayed using a standard ⁵¹Cr-release assay. Briefly, spleen cells are harvested from mice 14 days after the initial immunization or the booster immunization. Chromium release assays are performed as described in Lazdina, et al. (2003) *J. Gen. Virol.* 84:1-8, herein expressly incorporated by reference in its entirety. Single cell suspensions are prepared. 25 x10⁶ splenocytes are restimulated with 25 x10⁶ syngenic irradiated (20 Gy) splenocytes pulsed with 0.05 µM peptide, as previously described. Sandberg et al. (2000) *J. Immunol.* 165:25-33, herein expressly incorporated by reference in its entirety. Restimulation cultures are set in 12 ml complete RPMI medium (Gibco). After 5 days, effector cells are harvested and washed twice. RMA-S target cells (Karre et al. (1986) *Nature* 319:675-678) are pulsed with 50 µM peptide for 90 min at 5% CO₂ and 37°C. Serial dilutions of effector cells are incubated with

5 x 10³ chromium-labeled peptide pulsed RMA-S target cells in a final volume of 200 µl per well in 96-well plates. After a 4 hour incubation at 5% CO₂ and 37°C, 100 µl of supernatant is collected and the radioactivity is determined using a γ counter. The percentage of specific release is calculated according to the formula: (Experimental release – spontaneous release/total release- spontaneous release) x 100.

[0437] The results of the ⁵¹Cr-release assay is shown in **Figure 20A**. *i.m.* injection of HBcAg-pVAX1 elicits a cellular immune response. By contrast, immunization with HBcAg-pVAX1 via a gene gun does not elicit a cellular immune response. **Figure 20B**.

[0438] In another set of experiments, the presence of CTLs specific for the **SEQ ID NO:1014** is assayed using a standard ELISPOT assay to detect γ-IFN-secreting CTLs. Current Protocols in Immunology, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001 John Wiley & Sons, NY, N.Y.)

[0439] In still another set of experiments, the NS3/4A chimeric polypeptides encoded by the nucleic acids are used to immunize mice, using standard immunization procedures for polypeptides such as those disclosed in co-pending U.S. Patent Application No. 60/685,014, the contents of which is hereby expressly incorporated by reference in its entirety.

[0440] In contrast to HBcAg-pVAX1, nucleic acids encoding the NS3/4A peptide can effectively prime CTLs by both intra muscular and gene gun delivery. See, *e.g.*, co-pending U.S. Provisional Patent Application No. 60/685,014. To demonstrate that NS3/4A functions as a T-cell epitope carrier, chimeric NS3/4A nucleic acids that include the TCE encoded by **SEQ ID NO:1015**, or the encoded polypeptides, are tested for their ability to prime CTLs by both *i.m.* and gene gun delivery. The NS3/4A-pVAX vector described in **Example 1** is used to generate NS3/4A chimeric expression vectors containing in-frame fusions of **SEQ ID NO:1015** using standard cloning techniques. See, Ausubel et al., *supra*. The chimeric NS3/4A expression vectors contain in-frame fusions of **SEQ ID NO:1015** to the 5' end of the NS3/4A coding sequence; to the 3' end of the NS3/4A coding sequence, and within the NS3/4A coding sequence such that the epitope of **SEQ ID NO:1015** is between amino acids 181 and 182 of **SEQ ID NO: 36**, between amino acid residues 453 and 513 of **SEQ ID NO:36** (*e.g.*, **SEQ ID NO:1013**, which encodes the NS3/4A chimeric polypeptide of **SEQ ID NO:1012**) or in analogous positions in any NS3/4A polypeptide, or elsewhere

within the NS3/4A polypeptide. The chimeric NS3/4A nucleic acids are delivered to mice either intramuscularly or using a gene gun, as described herein. Specific CTL responses are measured using a ^{51}Cr -release assay or ELIspot assay.

[0441] The ability of the chimeric NS3/4A vectors to prime CTLs is similar whether the vector is administered intramuscularly or using a gene gun, demonstrating that presentation of epitopes in the context of NS3/4A effectively primes CTLs against the epitopes. This example also suggests that the NS3/4A platform is useful for generating immune responses to HBV TCEs that elicit immune responses in humans, (*e.g.*, **SEQ ID NO:351**).

[0442] The following example describes the generation and validation of immunogenic compositions that generate or enhance CTL priming to specific antigens.

EXAMPLE 20

[0443] Chimeric NS3/4A nucleic acid constructs encoding at least one TCE juxtaposed to or inserted within various positions along the NS3/4A polypeptide are made and assayed for their ability to prime an immune response to the TCE. Chimeric polypeptides encoded by the NS3/4A chimeric nucleic acids are also assayed for their ability to prime an immune response to the encoded TCE. A TCE to which a CTL response is desired (*e.g.*, any one of the TCEs presented herein, including **SEQ ID NOs: 221-271**, **SEQ ID NOs:809-1011**, and **SEQ ID NO:1014**) is selected. Using standard cloning techniques, the nucleic acid encoding the TCE (*e.g.*, any one of the TCEs presented herein, including **SEQ ID NOs: 221-271**, **SEQ ID NOs:809-1011**, and **SEQ ID NO:1014**) is cloned into the NS3/4A-pVAX vector described in **Example 1**, or an equivalent thereof (*e.g.*, an NS3/4A-pVAX vector wherein the NS3/4A sequence is selected from the group of **SEQ ID NOs: 572-808**) to generate a chimeric NS3/4A-pVAX vector. The chimeric NS3/4A-pVAX vectors encode chimeric NS3/4A polypeptides in which the TCE is juxtaposed to the N-terminus or C-terminus of the NS3/4A polypeptide, or is located within the NS3/4A polypeptide (*e.g.*, between amino acids 181 and 182 of **SEQ ID NO: 2**).

[0444] Plasmids that have been sequenced for accuracy are purified and prepared for use in immunization as described in **Example 19**. Alternatively, polypeptides encoded by said nucleic acids are expressed and used in immunizations as described in **Example 19**.

Mice are primed with the chimeric NS3/4A-pVAX nucleic acids intra muscularly (*i.m.*) or using a gene gun as described in **Example 19**, or by another method (e.g., using electroporation (Innovio, Oslo, Sweden) according to the manufacturer's instructions).

[0445] The priming of CTLs specific for the TCE (e.g., an epitope listed in **presented herein**, including **SEQ ID NOs: 221-271**, **SEQ ID NOs:809-1011**, and **SEQ ID NO:1014**) is assayed using a standard ⁵¹Cr-release assay or a standard ELISPOT assay to detect γ -IFN-secreting CTLs. Data from the ⁵¹Cr-release assay or the ELISPOT assay are used to determine preferred sites of insertion of the TCE within the NS3/4A-pVAX vector

[0446] Chimeric NS3/4A expression vectors contain in-frame fusions of TCEs (e.g., an epitope listed in **presented herein**, including **SEQ ID NOs: 221-271**, **SEQ ID NOs:809-1011**, and **SEQ ID NO:1014**) to the 5' end of the NS3/4A coding sequence; to the 3' end of the NS3/4A coding sequence, and within the NS3/4A coding sequence such that the TCE is between amino acids 181 and 182 of **SEQ ID NO: 36**, between amino acid residues 453 and 513 of **SEQ ID NO:36**, or in analogous positions in any NS3/4A polypeptide, or elsewhere within the NS3/4A polypeptide. The chimeric NS3/4A nucleic acids or encoded polypeptides are delivered to mice either intramuscularly or using a gene gun, as described herein. Specific CTL responses are measured using a ⁵¹Cr-release assay or ELISPOT assay as described in **Example 3**.

[0447] For each TCE, preferred sites of insertion within an NS3/4A nucleic acid, or juxtaposed to the NS3/4A nucleic acid are determined by comparing the immune responses generated by the chimeric nucleic acids or encoded polypeptides. Accordingly, provided herein are methods of making an immunogen that can include the steps of a) identifying a TCE against which an immune response is desired b) generating at least one chimeric NS3/4A nucleic acid in which the DNA sequence encoding the TCE is juxtaposed to or inserted within the NS3/4A sequence (e.g., **SEQ ID NO: 1**), and c) detecting the immune response generated by the chimeric NS3/4A nucleic acid or encoded polypeptide.

EXAMPLE 21

[0448] The Hepatitis B viral core protein (HBc) is an immunogen that stimulates the T cell response of an immunized host animal. See, e.g., U.S. Pat. No. 4,818,527, U.S. Pat. No. 4,882,145 and U.S. Pat. No. 5,143,726, all of which are hereby expressly incorporated by

reference in their entirety. In fact, the Hepatitis B core protein (HBcAg) has been shown to elicit a specific T-cell response in immunized mice. It is contemplated that DNA immunogens that are codon-optimized for expression in humans and which encode the HCV NS3/4A platform and fragments of HBcAg separated by NS3 protease cleavage sites will effectively prime HBcAg-specific CTLs, stimulate HBcAg-specific proliferative T cell responses, and induce production of HBcAg-specific antibodies in animals when these DNA immunogens are delivered by various DNA vaccination methodologies. In some embodiments, it is contemplated that the DNA immunogens, which are codon-optimized for expression in humans and which encode the HCV NS3/4A platform and fragments of HBcAg separated by NS3 protease cleavage sites will be more effective at priming HBcAg-specific CTLs, stimulating HBcAg-specific proliferative T cell responses, and inducing production of HBcAg-specific antibodies in animals than conventional DNA immunogens that encode HBcAg antigens and more effective than DNA immunogens that encode the NS3/4A platform and fragments of HBcAg without NS3 protease cleavage sites..

[0449] To determine the immunogenicity of codon-optimized DNA constructs encoding the HCV NS3/4A platform and fragments of HBcAg separated by NS3 protease cleavage sites and to compare the efficiency of these constructs with conventional HBcAg-containing constructs with and without the NS3/4A platform, several codon-optimized DNA constructs encoding the HCV NS3/4A platform and fragments of HBcAg separated by NS3 protease cleavage sites including antigenic sequences in various orientations are made (see SEQ ID NOs: 1174-1198 and Figure 1). Codon-optimized DNA constructs encoding only the HBcAg and/or fragments thereof or encoding the NS3/4A platform and the HBcAg and/or fragments thereof without NS3 protease cleavage sites are also made for comparison. Codon optimized DNA encoding the HCV NS3/4A platform and fragments of HBcAg in various orientations separated by NS3 protease cleavage sites are cloned into the pVAX1 expression vector (Invitrogen, Carlsbad, CA) or other suitable DNA vaccination vectors. Once the constructs are made, they are provided to animals by a DNA vaccination methodology (e.g., injection, electroporation, such as MedPulser®, or intranasal or transdermal delivery). Analysis of the presence and amount of HBcAg-specific CTLs can then be made before during and after several introductions of the constructs (e.g., an initial introduction followed by one, two, three, four, or five boosting events). It will be shown that

the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. It is also expected that the presence of shuffled HBcAg antigenic fragments (e.g., SEQ ID NOs: 1191-1198) within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof. The following describes these experiments in greater detail.

[0450] Plasmids containing the codon-optimized (human) DNA immunogens encoding the HCV NS3/4A platform and fragments of HBcAg separated by the NS3/4A protease cleavage site will be grown in BL21 E. coli cells, and sequenced for accuracy. Although the fragments of HBcAg are separated by the NS3/4A protease cleavage site, any NS3 protease cleavage site can be used (e.g., NS4A/B, NS4B/5A, and NS5A/B). The NS3/4A platform is separated from the fragments of HBcAg by an NS4A/B cleavage site, although any NS3 protease cleavage site can be used. In the construct, the NS3 platform is separated from the NS4A by the NS3/4A protease cleavage site, although any NS3 protease cleavage site can be used. Plasmids containing the conventional HBcAg sequence and/or fragments thereof will also be grown for comparison. Plasmid DNA used for in vivo vaccination is then purified using Qiagen DNA purification columns, according to the manufacturer's instructions (Qiagen GmbH, Hilden, FRG). The concentration of the resulting plasmid DNA is determined spectrophotometrically (Dynaquant, Pharmacia Biotech, Uppsala, Sweden) and the purified DNA is dissolved in sterile phosphate buffered saline (PBS) at a concentration of approximately 1 mg/ml.

[0451] Groups of eight to ten C57/BL6 mice or New Zealand rabbits are primed with an HBcAg-containing construct (see SEQ ID NOs: 1174-1198 and Figure 1) intranasally, transdermally, intra muscularly (i.m.), or using an electroporation device (e.g., MedPulser®).

[0452] If a transdermal or intranasal delivery is evaluated, an amount of plasmid DNA that is sufficient to deliver approximately 70µg-100µg of plasmid DNA per dose is formulated with the delivery vehicle. Animals are then provided the plasmid DNA one, two, three, four, or five times at monthly intervals. Prior to transdermal immunization, the delivery area is shaved.

[0453] If intramuscular injection is evaluated, animals are immunized i.m with approximately 70-100 µg plasmid DNA at the tibialis anterior (TA) muscle. 5 days prior to DNA immunization, animals may also be injected intramuscularly with 50 µl per TA muscle of 0.01 mM cardiotoxin (Latoxan) in 0/9% sterile saline.

[0454] When electroporation is evaluated, animals are immunized i.m with approximately 70-100 µg plasmid DNA at the tibialis anterior (TA) muscle and immediately after injection, the Medpulsar® is applied with a 0.5cm needle array set to deliver two 60 ms pulses of 246 V/cm to the injection site. In mice, one two needle electrode tip is used and when rabbits are used, one four needle electrode tip is used per injection per animal. The procedure can be repeated up to three times in mice and up to five times in rabbits at monthly intervals.

[0455] If gene gun delivery is performed, plasmid DNA is linked to gold particles according to protocols supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Prior to immunization, the injection area is shaved and the immunization is performed according to the manufacturer's protocol. Each injection dose by gene gun contains 4-100 µg of plasmid DNA. Immunizations are performed on weeks 0 and 4.

[0456] The presence of CTLs specific for HBcAg is then assayed using a standard ⁵¹Cr-release assay. Briefly, spleen cells are harvested from immunized animals 14 days after the initial immunization or a booster immunization. Chromium release assays are performed as described in Lazdina, et al. (2003) J. Gen. Virol. 84:1-8, herein expressly incorporated by reference in its entirety. Single cell suspensions are prepared. 25 x10⁶ splenocytes are restimulated with 25 x10⁶ syngenic irradiated (20 Gy) splenocytes pulsed with 0.05 µM peptide, as previously described. Sandberg et al. (2000) J. Immunol. 165:25-33, herein expressly incorporated by reference in its entirety. Restimulation cultures are set in 12 ml complete RPMI medium (Gibco). After 5 days, effector cells are harvested and washed twice. RMA-S target cells (Karre et al. (1986) Nature 319:675-678) are pulsed with 50 µM peptide for 90 min at 5% CO₂ and 37°C. Serial dilutions of effector cells are incubated with 5 x 10³ chromium-labeled peptide pulsed RMA-S target cells in a final volume of 200 µl per well in 96-well plates. After a 4 hour incubation at 5% CO₂ and 37°C, 100 µl of supernatant is collected and the radioactivity is determined using a γ counter. The percentage of specific release is calculated according to the formula: (Experimental release – spontaneous

release/total release- spontaneous release) x 100. The results of the ^{51}Cr -release assay will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will further show that the presence of shuffled HBcAg antigenic fragments (e.g., SEQ ID NOs: 1191-1198) within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0457] In another set of experiments, the presence of γ -IFN-secreting CTLs and T helper (Th) cells to HBcAg in spleenocyte or lymph node cultures will be evaluated using a commercially available ELISpot assay. (See Current Protocols in Immunology, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001 John Wiley & Sons, NY, N.Y.), herein expressly incorporated by reference in its entirety). By this approach, the number of γ -IFN-secreting CTLs or spots is determined at various concentrations of peptide. These experiments will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will further show that the presence of shuffled HBcAg antigenic fragments (e.g., SEQ ID NOs: 1191-1198) within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0458] In still another set of experiments, the proliferative responses to HBcAg in whole blood obtained from immunized animals is determined. An amount of whole blood is obtained from the animal (e.g., approximately 4ml from a rabbit), prior to the first vaccination and two weeks after each vaccination. The blood is collected in Heparin tubes and the plasma and peripheral mononuclear cells (PBMCs) are isolated by gradient centrifugation. The plasma is stored at -80 degrees Centigrade until analysis for HBcAg-specific antibodies. The presence and amount of antibodies specific for HBcAg in the various samples can be measured using an ELISA assay. The PBMCs are immediately assayed for in vitro proliferative recall responses using a standard 96h proliferation assay. (See Lazinda et al., J. Gen. Virol. 82:1299-1308 (2001), herein expressly incorporated by reference in its entirety.) In brief, microtiter plates are seeded with approximately 200,000

cells/well and the cells are incubated with media alone, phytohemagglutinin (PHA) or recombinant HbcAg. After 72 hours, radioactive thymidine is added and 16-24 hours later the cells are harvested. The proliferation is determined as radioactivity of the cells as the counts per minute (cpm) of cells incubated with the antigen divided by the CPM of the cells incubated with the media alone (sample to negative ration; S/N). Groups are compared by the mean S/N ratios at several time points). These experiments will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will also show that the presence of shuffled HBcAg antigenic fragments (e.g.,SEQ ID NOs: 1191-1198) within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0459] In yet another set of experiments, tumor inhibition assays will be carried out. Two weeks after the last immunization, mice will be challenged using tumor cells expressing the corresponding vaccine antigen, and protection against tumor growth will be measured. These experiments will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will also show that the presence of shuffled HBcAg antigenic fragments (e.g.,SEQ ID NOs: 1191-1198) within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0460] In still another set of experiments, quantification of HBcAg CTL responses will be measured by flow cytometry (Tetramers and Dimer-X). These experiments will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will also show that the presence of shuffled HBcAg antigenic fragments (e.g.,SEQ ID NOs: 1191-1198) within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

EXAMPLE 22

[0461] A similar methodology as that provided in EXAMPLE 21 can be applied to evaluate any DNA immunogen provided herein. More specifically, it is contemplated that DNA immunogens that are codon-optimized for expression in humans and which encode the HCV NS3/4A platform and one or more fragments of the antigens provided in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328, wherein said fragments are separated by NS3 protease cleavage sites will effectively prime antigen-specific CTLs, stimulate antigen-specific proliferative T cell responses, and induce production of antigen-specific antibodies in animals when these DNA immunogens are delivered by various DNA vaccination methodologies. Examples of antigen fragments of SEQ ID NOs: 1019-1021, SEQ ID NO: 1146, SEQ ID NOs: 1150-1166, SEQ ID NO: 1168, SEQ ID NO: 1170, and SEQ ID NO: 1172 separated by the NS3 protease cleavage site NS3/4A are presented in SEQ ID NOs: 1122-1145. Although the fragments in SEQ ID NOs: 1122-1145 are separated by the NS3/4A protease cleavage site, any NS3 protease cleavage site can be used (e.g., NS4A/B, NS4B/5A, and NS5A/B). Additionally, it is contemplated that DNA immunogens that are codon-optimized for expression in humans and which encode the HCV NS3/4A platform and a plurality of antigenic fragments from the antigens presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328, separated by NS3 protease cleavage sites, including antigenic sequences in various orientations as seen with the HBcAg from earlier examples, will also effectively prime antigen-specific CTLs, stimulate antigen-specific proliferative T cell responses, and induce production of antigen-specific antibodies in animals when these DNA immunogens are delivered by various DNA vaccination methodologies. In some embodiments, it is contemplated that the DNA immunogens, which are codon-optimized for expression in humans and which encode the HCV NS3/4A platform and one or more fragments of the antigens provided in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 separated by a NS3 protease cleavage site will be more effective at priming antigen-specific CTLs, stimulating antigen-specific proliferative T cell responses, and inducing production of antigen-specific antibodies in animals than conventional DNA immunogens that encode the antigens alone.

[0462] DNA constructs encoding the HCV NS3/4A platform and fragments of the antigens presented in SEQ ID NOs: 1019-1021, SEQ ID NOs: 1146-1173 and SEQ ID NOs:

1210-1328 separated by NS3 protease cleavage sites, including antigenic sequences in various orientations, are made. SEQ ID NOs: 1098-1121 presents codon optimized fragments of antigens presented in SEQ ID NOs: 1019-1021, SEQ ID NO: 1146, SEQ ID NOs: 1150-1166, SEQ ID NO: 1168, SEQ ID NO: 1170, and SEQ ID NO: 1172, wherein the fragments are separated by NS3/4A protease cleavage sites. Although the fragments are separated by NS3/4A protease cleavage sites, any NS3 protease cleavage site can be used. Additionally, although the fragments presented in SEQ ID NOs: 1098-1121 are configured in a naturally occurring order, separated by NS3 protease cleavage sites, fragments in various orientations, similar to the shuffled fragments of HBcAg in SEQ ID NOs: 1191-1198, are made. The shuffled fragments of the antigens presented in SEQ ID NOs: 1019-1021, SEQ ID NO: 1146, SEQ ID NOs: 1150-1166, SEQ ID NO: 1168, SEQ ID NO: 1170, and SEQ ID NO: 1172 are also separated by an NS3 protease cleavage site. Codon-optimized DNA constructs encoding only the fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 or encoding the NS3/4A platform and the fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 without NS3 protease cleavage sites are also made for comparison. Codon optimized DNA encoding the HCV NS3/4A platform and fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 in various orientations separated by NS3 protease cleavage sites are cloned into the pVAX1 expression vector (Invitrogen, Carlsbad, CA) or other suitable DNA vaccination vectors. Once the constructs are made, they are provided to animals by a DNA vaccination methodology (e.g., injection, electroporation, such as MedPulser®, or intranasal or transdermal delivery). Analysis of the presence and amount of antigen-specific CTLs can then be made before during and after several introductions of the constructs (e.g., an initial introduction followed by one, two, three, four, or five boosting events). It will be shown that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. It is also expected that the presence of shuffled antigenic fragments within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof. The following describes these experiments in greater detail.

[0463] Plasmids containing the codon-optimized (human) DNA immunogens encoding the HCV NS3/4A platform and fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 separated by the NS3/4A protease cleavage site will be grown in BL21 *E. coli* cells, and sequenced for accuracy. Although the fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 are separated by the NS3/4A protease cleavage site, any NS3 protease cleavage site can be used (e.g., NS4A/B, NS4B/5A, and NS5A/B). The NS3/4A platform is separated from the fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 by an NS4A/B cleavage site, although any NS3 protease cleavage site can be used. In the construct, the NS3 platform is separated from the NS4A by the NS3/4A protease cleavage site, although any NS3 protease cleavage site can be used. Plasmids containing codon-optimized nucleic acids encoding conventional fragments of antigen presented in SEQ ID NOs: 1016-1034, SEQ ID NOs: 1146-1173 and SEQ ID NOs: 1210-1328 will also be grown for comparison. Plasmid DNA used for *in vivo* vaccination is then purified using Qiagen DNA purification columns, according to the manufacturer's instructions (Qiagen GmbH, Hilden, FRG). The concentration of the resulting plasmid DNA is determined spectrophotometrically (Dynaquant, Pharmacia Biotech, Uppsala, Sweden) and the purified DNA is dissolved in sterile phosphate buffered saline (PBS) at a concentration of approximately 1 mg/ml.

[0464] Groups of eight to ten C57/BL6 mice or New Zealand rabbits are primed with an antigen-containing construct intranasally, transdermally, intra muscularly (*i.m.*), or using an electroporation device (e.g., MedPulser®).

[0465] If a transdermal or intranasal delivery is evaluated, an amount of plasmid DNA that is sufficient to deliver approximately 70µg-100µg of plasmid DNA per dose is formulated with the delivery vehicle. Animals are then provided the plasmid DNA one, two, three, four, or five times at monthly intervals. Prior to transdermal immunization, the delivery area is shaved.

[0466] If intramuscular injection is evaluated, animals are immunized *i.m.* with approximately 70-100 µg plasmid DNA at the tibialis anterior (TA) muscle. 5 days prior to DNA immunization, animals may also be injected intramuscularly with 50 µl per TA muscle of 0.01 mM cardiotoxin (Latoxan) in 0.9% sterile saline.

[0467] When electroporation is evaluated, animals are immunized *i.m* with approximately 70-100 µg plasmid DNA at the tibialis anterior (TA) muscle and immediately after injection, the Medpulser® is applied with a 0.5cm needle array set to deliver two 60 ms pulses of 246 V/cm to the injection site. In mice, one two needle electrode tip is used and when rabbits are used, one four needle electrode tip is used per injection per animal. The procedure can be repeated up to three times in mice and up to five times in rabbits at monthly intervals.

[0468] If gene gun delivery is performed, plasmid DNA is linked to gold particles according to protocols supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Prior to immunization, the injection area is shaved and the immunization is performed according to the manufacturer's protocol. Each injection dose by gene gun contains 4-100 µg of plasmid DNA. Immunizations are performed on weeks 0 and 4.

[0469] The presence of CTLs specific for antigen is then assayed using a standard ⁵¹Cr-release assay. Briefly, spleen cells are harvested from immunized animals 14 days after the initial immunization or a booster immunization. Chromium release assays are performed as described in Lazdina, et al. (2003) *J. Gen. Virol.* 84:1-8, herein expressly incorporated by reference in its entirety. Single cell suspensions are prepared. 25 x10⁶ splenocytes are restimulated with 25 x10⁶ syngenic irradiated (20 Gy) splenocytes pulsed with 0.05 µM peptide, as previously described. Sandberg et al. (2000) *J. Immunol.* 165:25-33, herein expressly incorporated by reference in its entirety. Restimulation cultures are set in 12 ml complete RPMI medium (Gibco). After 5 days, effector cells are harvested and washed twice. RMA-S target cells (Karre et al. (1986) *Nature* 319:675-678) are pulsed with 50 µM peptide for 90 min at 5% CO₂ and 37°C. Serial dilutions of effector cells are incubated with 5 x 10³ chromium-labeled peptide pulsed RMA-S target cells in a final volume of 200 µl per well in 96-well plates. After a 4 hour incubation at 5% CO₂ and 37°C, 100 µl of supernatant is collected and the radioactivity is determined using a γ counter. The percentage of specific release is calculated according to the formula: (Experimental release – spontaneous release/total release- spontaneous release) x 100. The results of the ⁵¹Cr-release assay will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves

immunogenicity. The assay will further show that the presence of shuffled antigenic fragments within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0470] In another set of experiments, the presence of γ -IFN-secreting CTLs and T helper (Th) cells to antigens in spleenocyte or lymph node cultures will be evaluated using a commercially available ELISpot assay. (See Current Protocols in Immunology, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001 John Wiley & Sons, NY, N.Y.), herein expressly incorporated by reference in its entirety). By this approach, the number of γ -IFN-secreting CTLs or spots is determined at various concentrations of peptide. These experiments will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will further show that the presence of shuffled antigenic fragments within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0471] In still another set of experiments, the proliferative responses to antigen in whole blood obtained from immunized animals is determined. An amount of whole blood is obtained from the animal (e.g., approximately 4ml from a rabbit), prior to the first vaccination and two weeks after each vaccination. The blood is collected in Heparin tubes and the plasma and peripheral mononuclear cells (PBMCs) are isolated by gradient centrifugation. The plasma is stored at -80 degrees Centigrade until analysis for antigen-specific antibodies. The presence and amount of antibodies specific for antigen in the various samples can be measured using an ELISA assay. The PBMCs are immediately assayed for *in vitro* proliferative recall responses using a standard 96h proliferation assay. (See Lazinda et al., J. Gen. Virol. 82:1299-1308 (2001), herein expressly incorporated by reference in its entirety.) In brief, microtiter plates are seeded with approximately 200,000 cells/well and the cells are incubated with media alone, phytohemagglutinin (PHA) or recombinant antigen. After 72 hours, radioactive thymidine is added and 16-24 hours later the cells are harvested. The proliferation is determined as radioactivity of the cells as the counts per minute (cpm) of cells incubated with the antigen divided by the CPM of the cells incubated with the media alone (sample to negative ration; S/N). Groups are compared by

the mean S/N ratios at several time points). These experiments assay will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will further show that the presence of shuffled antigenic fragments within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0472] In yet another set of experiments, tumor inhibition assays will be carried out. Two weeks after the last immunization, mice will be challenged using tumor cells expressing the corresponding vaccine antigen, and protection against tumor growth will be measured. These experiments will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will further show that the presence of shuffled antigenic fragments within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0473] In still another set of experiments, quantification of antigen CTL responses will be measured by flow cytometry (Tetramer and Dimer-X). These experiments assay will show that the presence of the HCV NS3/4A platform provides a more robust DNA immunogen, as compared to immunogens that lack the NS3/4A platform, and that the presence of one or more NS3/4A protease cleavage sites within the antigen also improves immunogenicity. The assay will further show that the presence of shuffled antigenic fragments within the antigen will provide a greater immune response than the unshuffled native antigen or fragments thereof.

[0474] Although the invention has been described with reference to embodiments and examples, it should be understood that various modification can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All of the patents, patent applications, and references cited herein are expressly incorporated by reference in their entireties.

EXAMPLE 23

[0475] Groups of C57/BL6 mice were immunized twice with 50 µg of either plasmid containing a codon optimized NS3/4A gene, a NS3/4A-Betv1 fusion gene containing a protease cleavage site between NS3 and NS4A as well as a protease cleavage site between the NS4A and the birch antigen (**SEQ ID NO: 1380**), or a NS3/4A-Betv1 fusion gene containing a protease cleavage site between NS3 and NS4A, a protease cleavage site between the NS4A and the birch antigen as well as two additional protease cleavage sites within the birch antigen (**SEQ ID NO: 1381**) using an electroporation device. Another group of mice were immunized twice with recombinant Betv1 protein (rBetv1) in Freund's incomplete adjuvant. The two immunizations were 4 weeks apart. The mice were sacrificed two weeks after the second immunizations and the lymph nodes and spleens of each group were collected and analyzed.

[0476] The presence of γ -IFN-secreting CTLs and T helper (Th) cells to antigens in spleenocyte or lymph node cultures were evaluated using a commercially available ELISpot assay. (*See Current Protocols in Immunology*, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001 John Wiley & Sons, NY, N.Y.), herein expressly incorporated by reference in its entirety). The spleen and lymph nodes from each group were pooled and immediately tested for the presence of NS3 and birch specific T cells. The ability of NS3-specific and birch-specific Th and CTLs to produce γ -IFN recalled by a concanavalin-A (con-A) as a positive control, media alone as a negative control, native HBcAg, recombinant HBcAg (rHBcAg), various concentrations of NS3/4A CTL peptide, various concentrations of rNS3, and various concentrations of rBetv1 was analyzed via the ELISpot. The results of the ELISpot assay are shown in **Figure 22**.

[0477] The NS3/4A-Betv1 (major Birch allergen) fusion genes showed that the NS3/4A clearly functions as an adjuvant for IFN- γ production. The NS3/4A-Betv1 fusion genes showed that the liberated NS3/4A-Betv1 fusion gene (**SEQ ID NO: 1380**) and fragmented NS3/4A-Betv1 fusion gene (**SEQ ID NO: 1381**) effectively prime IFN- γ producing Betv1-specific T cells two weeks after the second injection, where the recombinant Betv1 antigen fails to do. The data suggests the fragmented NS3/4A birch antigen fusion gene (**SEQ ID NO: 1381**) more effectively primes IFN- γ producing T-cells than the non-fragmented NS3/4A birch antigen fusion gene (**SEQ ID NO: 1380**).

EXAMPLE 24

[0478] Groups of C57/BL6 mice were immunized twice with 50 µg of either plasmid containing a codon optimized NS3/4A gene, a NS3/4A-Betv1 fusion gene containing a protease cleavage site between NS3 and NS4A as well as a protease cleavage site between the NS4A and the birch antigen (**SEQ ID NO: 1380**), or a NS3/4A-Betv1 fusion gene containing a protease cleavage site between NS3 and NS4A, a protease cleavage site between the NS4A and the birch antigen as well as two additional protease cleavage sites within the birch antigen (**SEQ ID NO: 1381**) using an electroporation device. Another group of mice were immunized twice with recombinant Betv1 protein (rBetv1) in Freund's incomplete adjuvant. The two immunizations were 4 weeks apart. The mice were bled two weeks after the second immunizations.

[0479] Dilutions of the sera were tested on ELISA plates coated with recombinant birch protein. A secondary antibody specific for IgE antibodies was used to detect bound antibody. The results are shown in **Figure 23**. The results show that when looking at the priming of IgE to Betv1, the DNA constructs do not prime IgE antibodies whereas the rBetv1 shows birch-specific IgE antibodies. The DNA constructs are thus not allergenic in that they prime a Th1 type response.

EXAMPLE 25

[0480] Groups of C57/BL6 mice were immunized twice with 50 µg using an electroporation device with either plasmid containing:

- a plasmid encoding a codon optimized NS3/4A gene,
- a naked pVAX-1 plasmid
- a plasmid containing a gene encoding HBcAg
- a plasmid containing an NS3/4A-HBcAg fusion gene encoding:
 - C1 (**SEQ ID NO: 1382**) having an active protease but no protease cleavage site anywhere on the fusion gene
 - C2 (**SEQ ID NO: 1383**) having an inactive protease and no protease cleavage site anywhere on the fusion gene

- C3 (**SEQ ID NO: 1384**) having an active protease and a protease cleavage site between NS3 and NS4A but no protease cleavage site anywhere else on the fusion gene
- C4 (**SEQ ID NO: 1385**) having an active protease and a protease cleavage site between NS3 and NS4A and a protease cleavage site between NS4A and HBcAg
- C5 (**SEQ ID NO: 1386**) having an active protease and a protease cleavage site between NS3 and NS4A a protease cleavage site between NS4A and HBcAg and 3 protease cleavage sites within the HBcAg which is in a naturally occurring order
- C5 (**SEQ ID NO: 1386**) having an active protease and a protease cleavage site between NS3 and NS4A a protease cleavage site between NS4A and HBcAg and 3 protease cleavage sites within the HBcAg which is in a naturally occurring order
- C6 (**SEQ ID NO: 1387**) having an active protease and a protease cleavage site between NS3 and NS4A a protease cleavage site between NS4A and HBcAg and 3 protease cleavage sites within the HBcAg which is in a non-naturally occurring order

[0481] The two immunizations were 4 weeks apart. The mice were sacrificed two weeks after the second immunizations and the lymph nodes and spleen from each mouse was collected.

[0482] The presence of CTLs specific for antigen was then assayed using a standard ⁵¹Cr-release assay. Briefly, the collected cells were harvested from immunized animals 14 days after the booster immunization. Chromium release assays were performed as described in Lazdina, et al. (2003) *J. Gen. Virol.* 84:1-8, herein expressly incorporated by reference in its entirety. Single cell suspensions are prepared. 25 x10⁶ splenocytes were restimulated with 25 x10⁶ syngenic irradiated (20 Gy) splenocytes pulsed with 0.05 μM peptide, as previously described. Sandberg et al. (2000) *J. Immunol.* 165:25-33, herein expressly incorporated by reference in its entirety. Restimulation cultures were set in 12 ml complete RPMI medium (Gibco). After 5 days, effector cells were harvested and washed twice. RMA-S target cells (Karre et al. (1986) *Nature* 319:675-678) were pulsed with 50 μM

peptide for 90 min at 5% CO₂ and 37°C. Serial dilutions of effector cells were incubated with 5×10^3 chromium-labeled peptide pulsed RMA-S target cells in a final volume of 200 μ l per well in 96-well plates. After a 4 hour incubation at 5% CO₂ and 37°C, 100 μ l of supernatant was collected and the radioactivity was determined using a γ counter. The percentage of specific release was calculated according to the formula: (Experimental release – spontaneous release/total release- spontaneous release) x 100. The results of the ⁵¹Cr-release assay is presented in **Figure 24**. NS3/4A clearly functions as an effective adjuvant for lytic CTL priming. The peak CTL levels for the non-fragmented HBcAg were higher compared to all other constructs.

EXAMPLE 26

[0483] C57BL/6 (H-2b) and BALB/c (H-2d) mice were obtained from B&K universal Sollentuna, Sweden, Charles River Laboratories, (Sulzfeld, Germany), and Taconic (Lilleskensved, Denmark), CD4 -/- knock-out mice were provided by the Animal Facility at Department of Microbiology, Tumor and Cell biology, Karolinska Institutet, Solna, Sweden. All mice were used at 6 to 8 weeks of age at the start of experiments and the local committee on animal ethics approved all experiments.

[0484] Recombinant particulate HBcAg encompassing residues 1-183 was produced in E.coli and purified as described in Billaud JN, Peterson D, Barr M, et al. Combinatorial approach to hepadnavirus-like particle vaccine design. J Virol 2005;79:13656-66 and Billaud JN, Peterson D, Schodel F, et al. Comparative antigenicity and immunogenicity of hepadnavirus core proteins. J Virol 2005;79:13641-55, herein incorporated by reference. Chicken egg albumin (Ovalbumin, e.g. OVA) was purchased from Sigma-Aldrich (Saint Louis, MO). A CpG containing oligonucleotide (TCC ATG ACG TTC CTG ACG TT **[SEQ ID NO. 1388]**; Cybergene AB, Huddinge, Sweden) was used for in vivo administration in combination with plasmid DNA vectors.

[0485] An HBcAg-derived MHC class II binding peptide composed of residues 120-140 (SFGVWIRTPPAYRPPNAPIL **[SEQ ID NO. 1389]**) and three different MHC class I binding peptides (HBcAg: MGLKFRQL **[SEQ ID NO. 1390]** and SYVNTNMGL **[SEQ ID NO. 1391]**, HCV NS3: GAVQNEVTI **[SEQ ID NO. 1392]**) was synthesized by standard techniques [28] by a multiple peptide synthesizer using 9-fluorenylmethoxy-carbonyl chemistry (Syro, MultiSynTech, Biochem, Germany).

[0486] Construction of a eukaryotic vector expressing HBcAg was described Lazdina U, Alheim M, Nystrom J, et al. Priming of cytotoxic T cell responses to exogenous hepatitis B virus core antigen is B cell dependent. *J Gen Virol* 2003;84:139-46, herein incorporated by reference in its entirety. The plasmid DNA used for in vivo injections was purified using Qiagen DNA purification columns according to the manufacturer's instructions (Qiagen GmbH, Hilden, FRG).

[0487] For DNA immunization C57BL/6 (WT), BALB/c and CD4^{-/-} knock-out mice on a C57BL/6 background were immunized by needle injection intramuscularly (i.m.) in the tibialis anterior (TA) muscle with 0.5 to 100µg of plasmid DNA encoding HBcAg or HCV NS3/4A (HBcAg-pVAX1; or NS3/4A-pVAX1) with or without in vivo electroporation as described in Ahlen G, Soderholm J, Tjelle TE, et al. In vivo Electroporation Enhances the Immunogenicity of Hepatitis C Virus Nonstructural3/4A DNA by Increased Local DNA Uptake, Protein Expression, Inflammation, and Infiltration of CD3⁺ cells. *J Immunol* 2007, herein incorporated by reference in its entirety, or transdermally by Gene gun (Bio Rad, Hercules, CA) with two to 18 micrograms of DNA Mice received booster doses every four weeks and were bled every second week. For detection of specific cellular immune responses mice were sacrificed 14 days after the last immunization. HBcAg specific antibody titers were determined by an in house enzyme-linked immuno sorbent assay (ELISA).

[0488] C57BL/6 mice were hydrodynamically injected with the HBcAg-encoding plasmid as described in Ahlen G, Nystrom J, Pult I, Frelin L, Hultgren C and Sallberg M. In Vivo Clearance of Hepatitis C Virus Nonstructural 3/4A-Expressing Hepatocytes by DNA Vaccine-Primed Cytotoxic T Lymphocytes. *J Infect Dis* 2005;192:2112-6 and. Ahlen G, Weiland M, Derk E, et al. Cleavage of the IPS-1/Cardif/MAVS/VISA does not inhibit T cell-mediated elimination of hepatitis C virus non-structural 3/4A-expressing hepatocytes. *Gut* 2008, herein incorporated by reference in their entirety. In brief, mice were immunized twice intramuscularly as described previously. Two weeks after last immunization a total volume of 1.5-1.8 mL of Ringer solution containing 100µg of HBcAg-DNA was injected intravenously in the tail vein within <10 seconds. After 24 and 48 hours mice were sacrificed and livers harvested.

[0489] HBcAg expression was detected in total liver homogenates essentially as described in Ahlen G, Soderholm J, Tjelle TE, et al. In vivo Electroporation Enhances the

Immunogenicity of Hepatitis C Virus Nonstructural3/4A DNA by Increased Local DNA Uptake, Protein Expression, Inflammation, and Infiltration of CD3+ cells. J Immunol and relin L, Brenndorfer ED, Ahlen G, et al. The hepatitis C virus and immune evasion: non-structural 3/4A transgenic mice are resistant to lethal tumour necrosis factor {alpha} mediated liver disease. Gut 2006;55:1475-83, herein incorporated by reference in their entirety. Liver tissue (100mg) was homogenized and analyzed by immunoprecipitation, followed by SDS-PAGE and Western blot. In brief, organ homogenates were lysed in 1ml of 0,15mol/L NaCl containing 50 mmol/L Tris, 1% Triton X-100, 1% Na-deoxycholate, and 1% SDS. The homogenates were immunoprecipitated with protein A sepharose and polyclonal mouse anti-HBc antibodies overnight at 4 °C on rotation. The washed pellets were re-suspended in SDS sample buffer, heated at 100 °C for 5 min before SDS-PAGE analysis on 4%-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and electrotransferred onto nitrocellulose membranes. HBc protein was detected using a rabbit anti-mouse HBcAg monoclonal antibody (DakoPatts, Glostrup, Denmark) with a chemiluminescence-linked Western blot kit (WesternBreeze, Invitrogen, Carlsbad, CA), in accordance with the manufacturer's protocol. Chemiluminescent signals were detected using the GeneGnome (Syngene, Cambridge, UK).

[0490] Detection of specific HBcAg antibodies and IgG isotype distribution was determined by solid phase ELISA as described in Hultgren C, Desombere I, Leroux-Roels G, et al. Evidence for a relation between the viral load and genotype and hepatitis C virus-specific T cell responses. J Hepatol 2004;40:971-8, herein incorporated by reference in its entirety.

[0491] Spleen cells were harvested and single cell suspensions were prepared and re-stimulated in vitro for five days as described in Lazdina U, Alheim M, Nystrom J, et al. Priming of cytotoxic T cell responses to exogenous hepatitis B virus core antigen is B cell dependent. J Gen Virol 2003;84:139-46 and Frelin L, Ahlen G, Alheim M, et al. Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4A gene. Gene Ther 2004;11:522-33, herein incorporated by reference in their entirety. In brief, 25×10^6 spleenocytes and 25×10^6 irradiated (2000 rad) syngenic spleenocytes were re-suspended in complete RPMI and re-stimulated with 0.05 μ M HBcAg93-100 or HCV NS3 MHC class I peptide. After five days of stimulation the in vitro cytolytic activity was measured by a standard four-hour ^{51}Cr -release assay, using 1×10^6

RMA-s target cells. The target cells were pulsed with 50 μ M HBcAg₉₃₋₁₀₀ or NS3 MHC class I peptide for 90 minutes and then labeled for one hour with 20-30 μ l of ^{51}Cr (5mCi/ml) (GE Healthcare, Uppsala, Sweden) and finally washed three times in PBS. During this period effector cells were harvested and washed. Cytolytic activity was determined at different effector to target (E:T) ratios, using 3 to 5 $\times 10^3$ ^{51}Cr -labeled target cells/well. All incubations were performed at 37 °C with 5% CO₂. The percentage of specific ^{51}Cr release was calculated according to the formula, [(experimental release-spontaneous release)/(total release-spontaneous release)] $\times 100$. Maximum release was calculated from supernatants of cells that were lysed by addition of Triton-X 100. Spontaneous release was determined from supernatants of cells incubated without effector cells. Results are shown as the mean percent specific lysis of triplicate values.

[0492] The frequency of HBcAg-specific CD8⁺ T cells was analyzed by ex vivo staining of spleen cells from DNA-immunized or non-immunized mice by using the HBcAg₉₃₋₁₀₀ MHC class I H-2Kb (MGLKFRQL [**SEQ ID NO. 1390**]) pentamer (ProImmune Ltd., Oxford, United Kingdom). In brief, freshly isolated spleen cells (1×10^6) were washed and re-suspended in PBS/1% FBS (FACS buffer) and incubated with R-PE labeled H-2Kb (MGLKFRQL [**SEQ ID NO. 1390**]) pentamer for 15 minutes in the dark at room temperature (22 °C). Cells were then washed and incubated with α -mouse CD16/32 antibodies (to block Fc binding, Bectin Dickinson Biosciences (BDB), San Jose, CA.) for 15 minutes in the dark at 4 °C. Following washing, cells were incubated with α -mouse CD8-FITC (clone KT15) and α -mouse CD19-PECy5 (clone 6D5) for 20 minutes in the dark at 4 °C. After two washes, cells were fixed in 2% paraformaldehyde in PBS for analysis by flow cytometry. Approximately 100,000 total events from each sample were acquired on a FACSCalibur flow cytometer (BDB) using the CellQuest software. From a live lymphocyte gate, CD19-positive events were excluded, and the remaining cells were gated for CD8 expression. Frequency of HBcAg H-2Kb (MGLKFRQL [**SEQ ID NO. 1390**]) positive events within this population was determined.

[0493] ELISpot assays were performed essentially as previously described in Ahlen G, Soderholm J, Tjelle TE, et al. In vivo Electroporation Enhances the Immunogenicity of Hepatitis C Virus Nonstructural3/4A DNA by Increased Local DNA Uptake, Protein Expression, Inflammation, and Infiltration of CD3+ cells. J Immunol 2007

and oderholm J, Ahlen G, Kaul A, et al. Relation between viral fitness and immune escape within the hepatitis C virus protease. *Gut* 2006;55:266-74, herein incorporated by reference in their entirety. In brief, nitrocellulose bottom 96-well plates (MAIPSWU10, Millipore Co., Bedford, MA) were coated with γ -IFN mAb (anti- γ -IFN AN18, Mabtech AB, Stockholm, Sweden) over-night at 4 °C. The following day re-stimulation was performed using 2×10^5 spleenocytes and lymphocytes per well with serial dilutions of proteins (ranging from 10 μ g/mL to 0.1 μ g/mL;), or synthetic peptides (ranging from 20 μ g/mL to 0.002 μ g/mL), or Phytohemagglutinin (PHA; 4 μ g/mL), or Concanavalin A (Con A; 2 μ g/mL). Both PHA-L and ConA was purchased from Sigma-Aldrich, Saint Louis, MO. The plates were left undisturbed for 40 to 44 hours at 37°C in a humidified atmosphere with 5% CO₂. Production of γ -IFN was detected by a biotin-conjugated anti- γ -IFN mAb (R4-6A, Mabtech AB, Stockholm, Sweden), streptavidin-alkaline phosphatase (Mabtech AB, Stockholm, Sweden) and BCIP/NBT substrate solution (Bio-Rad Laboratories, Richmond, CA). The number of spots was scored using the Aid ELISpot reader system Version 2.6 (Autoimmun Diagnostika, Germany).

[0494] Endogenously produced HBcAg is effective in priming a production of antibodies to HBcAg (anti-HBc) (Figure 25a). An i.m. injection of 100 μ g of wild-type (wt) HBcAg-DNA primed anti-HBc titres of >10.000 at four weeks after the first injection (Figure 25a). In contrast, transdermal delivery of 50-fold lower doses required at least two injections to reach titres >1.000 (Figure 25b). The addition of CpG oligonucleotides improved the antibody titres after transdermal delivery by around 10-fold, without altering the IgG-subclass profile (Figure 25b). An interesting observation was that the IgG2b-dominated subclass distribution of anti-HBc was maintained regardless of the delivery route or adjuvants, and this perfectly mimics the IgG subclass distribution seen after immunization with exogenous HBcAg. Thus, the ability of HBcAg to preferentially induce IgG2b appears to be an intrinsic property of HBcAg.

[0495] We next tested the immunogenicity of endogenous HBcAg by titrating the immunogen dose for i.m. delivery. We found that endogenous HBcAg rapidly lost its immunogenicity with decreased DNA doses at two weeks after a single immunization (Figure 25c). This could be corrected by adjuvanting the DNA injection by in vivo EP, which improved the immunogenicity by around 10-fold (Figure 25c). A wild-type HBcAg gene

only induces significant levels of anti-HBc at high DNA doses, or at lower doses when delivery is supported by different adjuvants.

[0496] To characterize the ability of wtHBcAg-DNA to prime specific CTLs at high and low doses we used i.m. and transdermal delivery using the gene gun since these approaches works very well in our hands with the HCV NS3/4A gene. Two i.m. injections with 100 µg wtHBcAg- or wtHBcAg-DNA prime detectable CTLs, whereas the same plasmids delivered transdermally fails to do the same (Figures 26a to 26d). We then increased the number of immunizations to three with the 100 µg dose i.m. (Figures 26e and 26g), or 2 µg dose transdermally (total dose 6 µg) but still failed to detect CTLs by transdermal delivery (Figure 26f). We next increased the dose to 6 µg and gave three injections (total dose 18 µg), and this induced detectable CTLs in two mouse strains (Figures 26g to 26j). However, this contrasts the ease by which a single transdermal immunization with a wtNS3/4A (Figure 26l) or coNS3/4A gene (Figure 26n), induces detectable CTLs, and at the same levels as when plasmids are given 100 µg i.m. (Figures 26k and 26m). Thus, the weak ability of HBcAg-DNA to prime CTLs at low DNA doses suggests that native HBcAg is a comparatively poor inducer of specific CTLs.

[0497] We next determined the DNA doses needed for an effective priming of HBcAg-specific CTLs by the i.m. route. HBcAg-DNA can induce CTLs at high doses, usually 50-100 µg per injection in a mouse. We could now show that endogenous HBcAg, surprisingly rapid, lost its ability to prime CTLs as determined by lysis of peptide loaded target cells (Figures 27a to 27h), by direct *ex-vivo* pentamer staining (Figure 27i) and by IFN-γ production (Figure 28). HBcAg-specific CTLs were only detected at the 5 µg dose when adjuvanting the i.m. injection by in vivo EP (Figures 27 and 28). Single doses lower than 5 µg delivered i.m. with (Figures 27f and 28f) or without (27c and 28c) in vivo EP, or with the gene gun (27g) completely failed to prime detectable CTLs. This is distinct from the HCV NS3/4A protein, which effectively induces CTLs by many routes of delivery and at low doses.

[0498] We then tested whether the CTLs primed by high doses of HBcAg DNA given i.m. were functional in vivo using transiently transgenic mice. In this model the clearance of antigen-expressing hepatocytes is completely dependent of CD8+ CTLs. The transient expression of HBcAg in hepatocytes is generated by a hydrodynamic injection of

HBcAg-DNA and was monitored at 24 and 48 hours by western blot (Figure 28i). At 24 hours after hydrodynamic injection of HBcAg-DNA six out of eight mice had detectable hepatic expression of HBcAg, regardless if CTLs had been primed or not (Figure 28i). Within 48 hours after the hydrodynamic injection, HBcAg expression was cleared in vaccinated mice (Figure 28i; 6/8 vs. 0/7, $p < 0.01$; Fisher's exact test). In the non-vaccinated mice no statistical evidence of clearance was found when comparing HBcAg expression at 24 and 48 hours (Figure 4i; 6/8 vs. 4/7, not significant, Fisher's exact test). In conclusion, two vaccinations of 100 μ g HBcAg-DNA delivered i.m. primes in vivo functional CTLs that enter the liver and eliminate HBcAg-expressing hepatocytes, a desired feature of a therapeutic vaccine. This confirms that the CTLs primed by the high dose DNA vaccination recognized endogenously produced hepatic HBcAg presented within the context of hepatic MHC class I molecules in vivo.

[0499] We examined the role of CD4⁺ T cells in the priming of HBcAg-specific CTLs. Wild-type and CD4^{-/-} mice were immunized once with 100 μ g HBcAg-DNA and the appearance of HBcAg-specific IFN γ -producing CTLs was determined at four and 17-days after immunization. This showed that HBcAg-specific IFN γ -producing CTLs were not detectable *ex-vivo* at day four, but became detectable at day 17 (Figures 29a and 29b). The corresponding CTL responses failed to appear in the CD4^{-/-} mice (Figures 29c and 29d). This suggests that the priming of HBcAg-specific CTLs, unlike the priming of NS3/4A-specific CTLs are dependent on CD4⁺ T helper cells.

[0500] The previous sets of experiments have shown that low doses of HBcAg-DNA given i.m. or by transdermal delivery failed to effectively prime IFN γ producing T cells. The last experiment highlighted the importance of CD4⁺ T helper cells in this event and we therefore hypothesized that the presence of an unlimited amount of HBcAg-specific T helper cells could improve CTL priming. To test this we primed H-2b mice with a peptide representing the dominant CD4⁺ T helper epitope at residues 120-140 of HBcAg nine days prior to the DNA immunization. This approach was found to improve humoral responses during genetic immunization with a retroviral vector. Again, one or two high doses of HBcAg-DNA delivered i.m. primed HBcAg-specific IFN γ -producing T helper cells and CTLs (Figures 29e and 29i), whereas low doses delivered transdermally failed to do the same (Figures 29f and 29j). Priming with the HBcAg-derived T helper peptide effectively induced

HBcAg-specific IFN γ -producing T helper cells that were recalled *in vitro* by both the peptide itself and recombinant HBcAg (Figures 29g and 295k). However, the presence of unlimited amounts of HBcAg-specific IFN γ -producing T helper cells was not able to correct the inability of transdermal delivery of low doses of HBcAg-DNA to prime CTLs (Figures 29h and 29l). Thus, despite that endogenously produced native HBcAg is dependent on CD4 $^{+}$ T help for the priming of CTLs, it seems that only improving the T helper function does not sufficiently promote the priming of CTLs when using low doses of HBcAg-DNA.

[0501] We had found that improving the priming environment by CpG adjuvant or by providing T help did not rescue the inability of low doses of wtHBcAg DNA to prime CTLs. However, *in vivo* electroporation, which improves DNA uptake, antigen expression, and local inflammation had a beneficial effect. We therefore further improved on HBcAg expression by codon optimization of the gene. We now found that this also improved the priming of HBcAg-specific T cells in several ways. First, the levels of IFN γ -producing T helper cells and CTLs was greatly improved at the 5 μ g dose, but not at the 50 or 0.5 μ g dose (Figure 30). This was reproduced as an increased number to HBcAg-specific CTLs as determined by tetramer staining (Figure 31) and by the levels of CTL lysis of peptide-loaded target cells (Figure 32).

[0502] At the antibody level, all data were reiterated with improvement provided by the codon optimization resulting of the priming of low levels of anti-HBc also at the 0.5 μ g dose (Figure 32). Thus, codon optimization has an additional beneficial effect on the immunogenicity of endogenous HBcAg but still fails help in the priming of detectable T cell responses at low doses.

[0503] HBcAg is an unusual antigen. When expressed *in vitro* HBcAg spontaneously forms capsid-like particles. These capsids can bind to the naive B cell receptor through a non-canonical binding and use naive B-cells as the primary APC. The efficiency of the B cell as the primary APC for exogenous HBcAg is most likely explained by the cross-linking surface-bound Ig, whereby the B cells matures into an activated APC. Exogenous HBcAg has been found to be highly immunogenic on a B and T helper cell level and can transfer this immunogenicity to foreign sequences that are inserted in to the tip of the protruding spike. Although it has been shown that genetic immunization with HBcAg, i.e. endogenously produced HBcAg, clearly induce immune responses including antibodies, T

helper cells and CTLs, little has been done to quantify its intrinsic immunogenicity. Surprisingly, HBcAg is a comparatively poor CTL-inducer as an endogenously produced antigen.

[0504] We used HBcAg-DNA as a model, a native HBcAg gene expressing a native full-length HBcAg protein that assembles into particles. We immediately found that, although HBcAg seems quite immunogenic at a first glance this is certainly not the case when the levels of DNA are reduced. This contrasts our previous findings with HCV NS3/4A gene, which is highly immunogenic in its native or codon optimized form. In particular, NS3/4A can prime CTLs at single DNA doses of less than 1 µg, whereas HBcAg is completely unable to do this regardless of the presence of adjuvants or increase antigen expression.

[0505] First, the immunogenicity of HBcAg-DNA drops rapidly between 50 µg and 5 µg doses of DNA when delivered *i.m.*, even if *in vivo* EP is used as an adjuvant. HCV NS3/4A-DNA effectively primes specific CTLs at a single 0.5 µg dose when delivered by *in vivo*. HBcAg DNA fails to activate IFNγ-producing T helper cells and CTLs at *i.m.* doses below 5 µg even with *in vivo* EP and a codon optimized gene. HCV NS3/4A-DNA again effectively primes these responses down to 0.5 µg. Thus, when delivered *i.m.*, with or without *in vivo* EP, HBcAg-DNA is at least 10- to a 100-fold less effective in T cell priming as compared to HCV NS3/4A. With respect to other routes of administration, HBcAg-DNA was unable to induce CTLs when administered transdermally thrice up to 2 or 4 µg doses (6 or 12 µg total dose; data not shown), whereas the NS3/4A gene primes *in vivo* functional CTLs transdermally after a single dose of 2 µg. HBcAg-DNA eventually induces HBcAg-specific CTLs when administered transdermally thrice at 6 µg doses (18 µg total dose), thus, at least 9-fold less efficient than NS3/4A-DNA when delivered by the gene gun.

[0506] To better understand the inability of low doses of HBcAg-DNA to prime specific CTLs, we added CpG ODNs and pre-primed HBcAg-specific Th cells. Unlike our own experience, others have been proposed that gene gun immunization may favor a T helper-2-like response shown by Feltquate DM, Heaney S, Webster RG and Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 1997;158:2278-84 and. McCluskie MJ, Brazolot Millan CL, Gramzinski RA, et al. Route and method of delivery of DNA vaccine influence immune

responses in mice and non-human primates. Mol Med 1999;5:287-300, herein incorporated by reference in their entirety. CpG oligonucleotides (ODNs) has been shown to sometimes, but not always, shift a gene-gun-mediated DNA immune response from Th2 towards Th1, as shown in Schirmbeck R, Reimann J. Modulation of gene-gun-mediated Th2 immunity to hepatitis B surface antigen by bacterial CpG motifs or IL-12. Intervirology 2001;44:115-23 and Weiss R, Scheiblhofer S, Freund J, Ferreira F, Livey I and Thalhamer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rides the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. Vaccine 2002;20:3148-54, herein incorporated by reference in their entirety. Thus, in an attempt to override the potential Th2-bias of transdermal delivery, we coated gold beads with a mix of plasmid DNA and immune stimulating CpG ODNs. Although the presence of CpG ODNs improved the immunogenicity evidenced by a 10-fold increase in anti-HBc, it could not correct the inability of the low doses of HBcAg-DNA delivered by the gene gun to prime HBcAg-specific CTLs. We found that the priming of HBcAg-specific CTLs was highly dependent of CD4+ T helper cells. This prompted us to address if the presence of an unlimited amount of HBcAg-specific Th cells could correct the inability of low doses of HBcAg-DNA to prime CTLs. This was not the case, since activation of IFN γ -producing HBcAg-specific Th cells using a synthetic peptide, prior to delivery of low doses of HBcAg-DNA failed to improve the priming of HBcAg-specific CTLs. Thus, HBcAg seems to have an intrinsically poor ability to prime CTLs, and this is quite unexpected.

[0507] We did find that increasing expression levels a step at a time, by codon optimizing the HBcAg gene and by introducing *in vivo* electroporation assisted delivery, the efficiency by which HBcAg induced CTLs improved. Again, and very surprisingly, despite all these measures was HBcAg unable to effectively prime CTLs at low DNA doses. This is an unexpected feature of HBcAg.

[0508] Some have tried to explain the high immunogenicity of exogenous HBcAg by the presence of contaminants or by immuno stimulating sequences. However, it is well known that in particular *E. coli* expressed HBcAg can contain high levels of lipopolysaccharide, and no relation has been shown between the *in vivo* immunogenicity and levels of LPS. Importantly, in HBV infected humans, who come into contact with both endogenous (intracellular) and exogenous (released by cell turnover/leakage/killing) HBcAg,

extreme levels of anti-HBc are generated, which suggests that HBcAg is highly immunogenic in humans in the absence of LPS. Therefore, the high level of immunogenicity exerted by exogenous HBcAg is most likely explained by a number of factors. First, HBcAg can effectively activate and use B cells as a primary APC. Second, multimeric antigens often have a high intrinsic immunogenicity towards a humoral immunity. Third, HBcAg can bind RNA, which can act as an adjuvant. Thus, one would expect that endogenous HBcAg would be an equally potent immunogen. The most immunogenic property of endogenous HBcAg seems to be its ability to rapidly prime anti-HBc, albeit this ability drops of quite rapidly at lower DNA doses. The least immunogenic property of endogenous HBcAg is its ability to prime CTLs. This requires high expression levels of HBcAg mediated by high levels of HBcAg-DNA and/or improved uptake and expression mediated by *in vivo* EP. Only improving the priming environment by administration by different routes, by the addition of CpG adjuvants or by improving the T helper function does not improve CTL priming. Thus, the only herein clearly identified factor that improves CTL priming is increased endogenous levels of HBcAg.

EXAMPLE 27

[0509] A Codon optimized HBcAg (nucleotide sequence containing restriction sites) that can be used with the embodiments described herein (e.g., genetic immunization with or without NS3/4A, mutant or modified NS3/4A, or in the absence of such enhancing sequences with or without electroporation techniques or CPG nucleotides) is shown below:

[0510]

_____G↓AATTTCGCACCATGGACATCGACCCCTACAAGGAGTTCGGCG
 CCACCGTGGAGCTGCTGAGCTTCCTGCCCAGCGACTTCTTCCCCAGCGTGAGAGA
 CCTGCTGGACACCGCCAGCGCCCTGTACAGAGAGGCCCTGGAGAGCCCCGAGCA
 CTGCAGCCCCCACCACCGCCCTGAGACAGGCCATCCTGTGCTGGGGCGAGCT
 GATGACCCTGGCCACCTGGGTGGGCGTGAACCTGGAGGACCCCGCCAGCAGAGA
 CCTGGTGGTGAGCTACGTGAACACCAACATGGGCCTGAAGTTCAGACAGCTGCT
 GTGGTTCCACATCAGCTGCCTGACCTTCGGCAGAGAGACCGTGATCGAGTACCT
 GGTGAGCTTCGGCGTGTGGATCAGAACCCCCCCCCGCTACAGACCCCCCAACGC
 CCCCATCCTGAGCACCTGCCCCGAGACCACCGTGGTGAGAAGAAGAGGCAGAAG
 CCCCAGAAGAAGAACCCCCAGCCCCAGAAGAAGAAGCCAGAGCCCCAGAA

GAAGAAGAAGCCAGAGCAGAGAGAGCCAGTGCTAGT↓CTAGA [SEQ ID NO. 1393].

[0511] G↓AATTC = EcoRI site

T↓CTAGA = XbaI site

GCACCATGG = Kozak sequence

ATG = Start codon

TAG = Stop codon

[0512] The coHBcAg gene was inserted into the pVAX1 plasmid backbone, where the protein-expression was driven by the CMV promoter. Of course, many other expression plasmids and promoters can be used.

EXAMPLE 28

[0513] Groups of C57/BL6 mice were immunized intra muscularly once with 50 µg of a plasmid expressing a codon optimized HBcAg gene, or a plasmid expressing an HBcAg gene of a wild types sequence. Two weeks later venous blood was taken and the levels of antibodies to HBcAg were determined by an enzyme immuno assay as Lazdina, et al. (2003) *J. Gen. Virol.* 84:1-8, herein expressly incorporated by reference in its entirety. In brief, 96-well plates were coated with sodiumcarbonate buffer, pH 9.6 containing 1 µg/mL of recombinant HBcAg over-night at +4°C. The plates were washed and then blocked by phosphate buffered saline containing 2% bovine serum albumine for 2 hours at room temperature. Dilutions of mouse sera were then added and incubated at +37°C for 90 minutes. The plates were washed and bound mouse antibodies were indicated by a an enzyme labeled goat anti-mouse antisera. The plates were washed and developed by the addition of a substrate. The absorbancies were determined spetrophotometrically. The priming of cytotoxic T cell responses to exogenous hepatitis B core antigen (HBcAg) is B cell dependent. *J Gen Virol* 84: 139-146). At two weeks the mice were sacrificed and the T cell responses were determined. The mice were sacrificed two weeks after the second immunizations and the lymph nodes and spleen from each mouse was collected. The presence of CTLs specific for antigen was then assayed using a standard ⁵¹Cr-release assay.

Briefly, the collected cells were harvested from immunized animals 14 days after the booster immunization. Chromium release assays were performed as described in Lazdina, et al. (2003) *J. Gen. Virol.* 84:1-8, herein expressly incorporated by reference in its entirety. Single cell suspensions are prepared. 25×10^6 splenocytes were restimulated with 25×10^6 syngenic irradiated (20 Gy) splenocytes pulsed with 0.05 μM peptide, as previously described. Sandberg et al. (2000) *J. Immunol.* 165:25-33, herein expressly incorporated by reference in its entirety. Restimulation cultures were set in 12 ml complete RPMI medium (Gibco). After 5 days, effector cells were harvested and washed twice. RMA-S target cells (Karre et al. (1986) *Nature* 319:675-678) were pulsed with 50 μM peptide for 90 min at 5% CO_2 and 37°C. Serial dilutions of effector cells were incubated with 5×10^3 chromium-labeled peptide pulsed RMA-S target cells in a final volume of 200 μl per well in 96-well plates. After a 4 hour incubation at 5% CO_2 and 37°C, 100 μl of supernatant was collected and the radioactivity was determined using a γ counter. The percentage of specific release was calculated according to the formula: $(\text{Experimental release} - \text{spontaneous release} / \text{total release} - \text{spontaneous release}) \times 100$. The levels of antibody and CTL responses were compared between the groups receiving plasmids containing the wild-type and the codon-optimized HBcAg genes.

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a hepatitis B virus core antigen (HBcAg) that is codon optimized for expression in humans.
2. The composition of Claim 1, wherein said nucleic acid is **SEQ ID NO. 1393**.
3. The composition of Claim 1 or 2, further comprising a nucleic acid encoding a hepatitis C virus (HCV) NS3/4A peptide.
4. The composition of any one of Claims 1-3 wherein said nucleic acid further comprises a nucleic acid encoding a hepatitis C virus (HCV) NS3 protease cleavage site, wherein said HCV NS3 protease cleavage site is joined at a position that is not naturally occurring.
5. The composition of Claim 4, wherein said HCV NS3 protease cleavage site comprises the sequence: SADLEVVTTSTWVLVGGVL.
6. The composition of Claim 4, wherein said HCV NS3 protease cleavage site comprises the sequence: DMEECSQHLPYIEQG.
7. The composition of any one of Claims 3-6, wherein said NS3/4A peptide comprises a mutation that enhances protease activity.
8. The composition of Claim 7, wherein said mutation is selected from the group consisting of Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly, Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, and Thr177Ala.
9. The composition of any one of Claims 3-8, wherein the nucleic acid sequence is codon optimized for expression in humans.
10. A nucleic acid encoding a plurality of antigenic fragments of a hepatitis B virus core antigen (HBcAg) assembled in a non-naturally occurring order, wherein said nucleic acid is codon optimized for expression in humans.
11. The composition of Claim 10, further comprising a nucleic acid encoding a hepatitis C virus (HCV) NS3/4A peptide.
12. The composition of any one of Claims 10 or 11 wherein said nucleic acid further comprises at least one nucleic acid encoding a hepatitis C virus (HCV) NS3 protease

cleavage site, wherein said HCV NS3 protease cleavage site is joined at a position that is not naturally occurring.

13. The composition of Claim 10 or 11 wherein said nucleic acid further comprises a nucleic acid encoding a hepatitis C virus (HCV) NS3 protease cleavage site between each antigenic fragment of HBcAg.

14. The composition of Claim 12 or 13, wherein said HCV NS3 protease cleavage site comprises the sequence: SADLEVVTSTWVLVGGVL.

15. The composition of Claim 12 or 13, wherein said HCV NS3 protease cleavage site comprises the sequence: DMEECSQHLPYIEQG.

16. The composition of any one of Claims 11-15, wherein said NS3/4A peptide comprises a mutation that enhances protease activity.

17. The composition of Claim 16, wherein said mutation is selected from the group consisting of Tyr6Ala, Arg11Ala, Leu13Ala, Leu14Ala, Glu30Ala, Cys52Ala, Gly58Ala, Ala59Gly, Ile64Ala, Ile64Ala, Gln73Ala, Thr76Ala, Pro86Ala, Ala111Gly, Gly 122Ala, Tyr 134Ala, Lys 136Ala, Gly 141Ala, Val158Ala, Arg161Ala, Ala166Gly, and Thr177Ala.

18. The composition of any one of Claims 11-17, wherein said nucleic acid is codon optimized for expression in humans.

19. A method of using any one or more of the compositions embodied in the aforementioned claims to induce an immune response in a subject comprising:

identifying a subject in need of an immune response to HBcAg; and

providing any one or more of the compositions of Claims 1-18 to said subject.

20. The method of Claim 19, further comprising measuring the induction of an immune response to said antigen.

21. The method of Claim 19 or 20, wherein said subject suffers from a hepatitis B infection.

22. The method of any one of Claims 19 or 20, wherein the identification is performed by clinical or diagnostic evaluation.

23. The method of any one of Claims 19 or 20, wherein said composition is provided by injection, DNA vaccination, transdermal application, or electroporation.

24. The method of Claim 19, further comprising providing an electrical stimulation before, during, or after providing the composition to said subject.

25. The method of any one of claims 19-24, wherein said composition is provided by a Medpulser®.

26. Use of the composition of any one of Claims 1-18 to induce an immune response to Hepatitis B Virus (HBV) in a subject.

27. Use of the composition of any one of Claims 1-18 to prepare a medicament for the purpose of inducing an immune response to HBV.

28. An immunogenic composition comprising any one or more of the compositions of Claims 1-18.

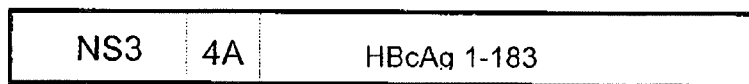


FIGURE 1A

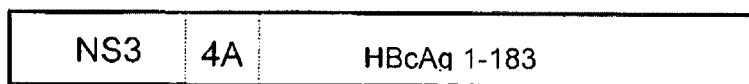


FIGURE 1B

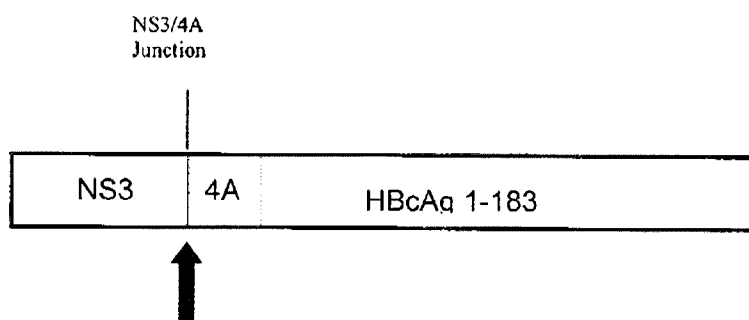


FIGURE 1C

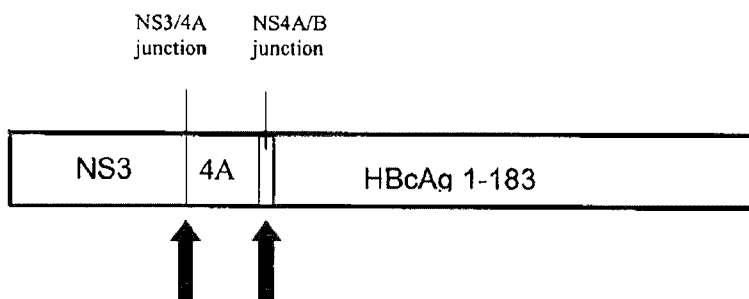


FIGURE 1D

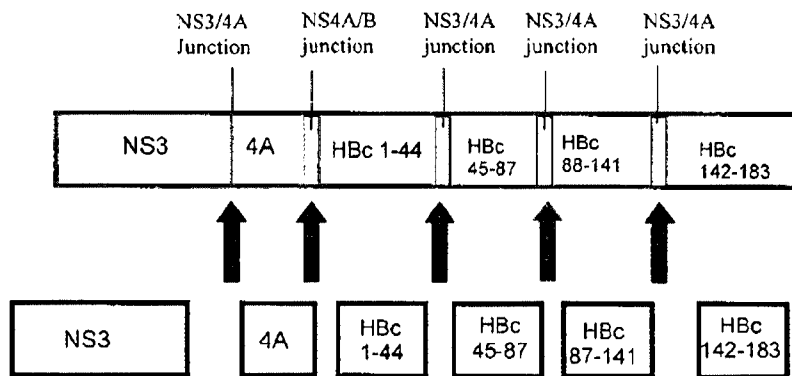


FIGURE 1E

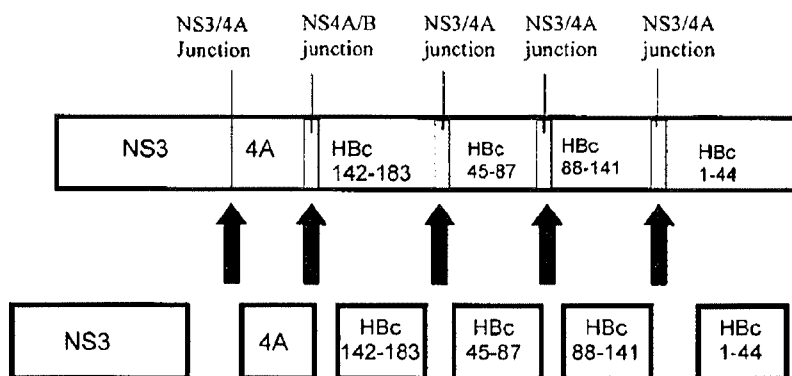


FIGURE 1F

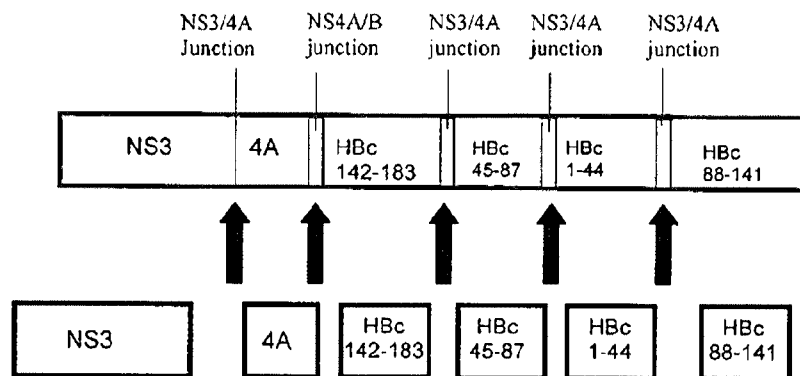


FIGURE 1G

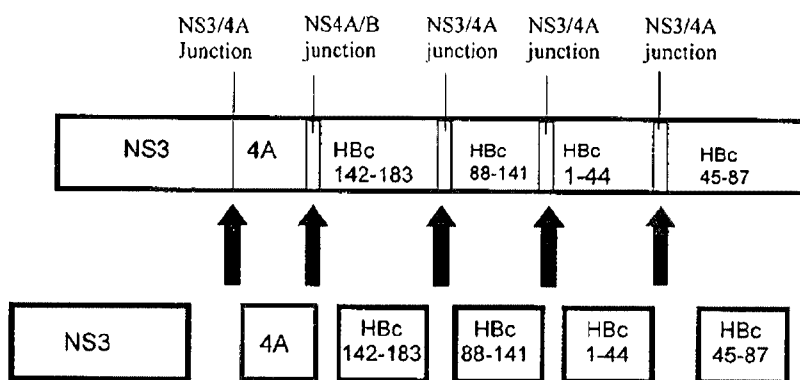


FIGURE 1H

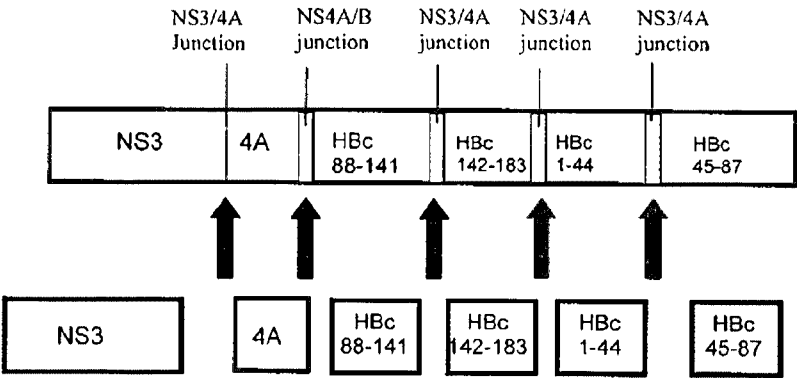


FIGURE 1I

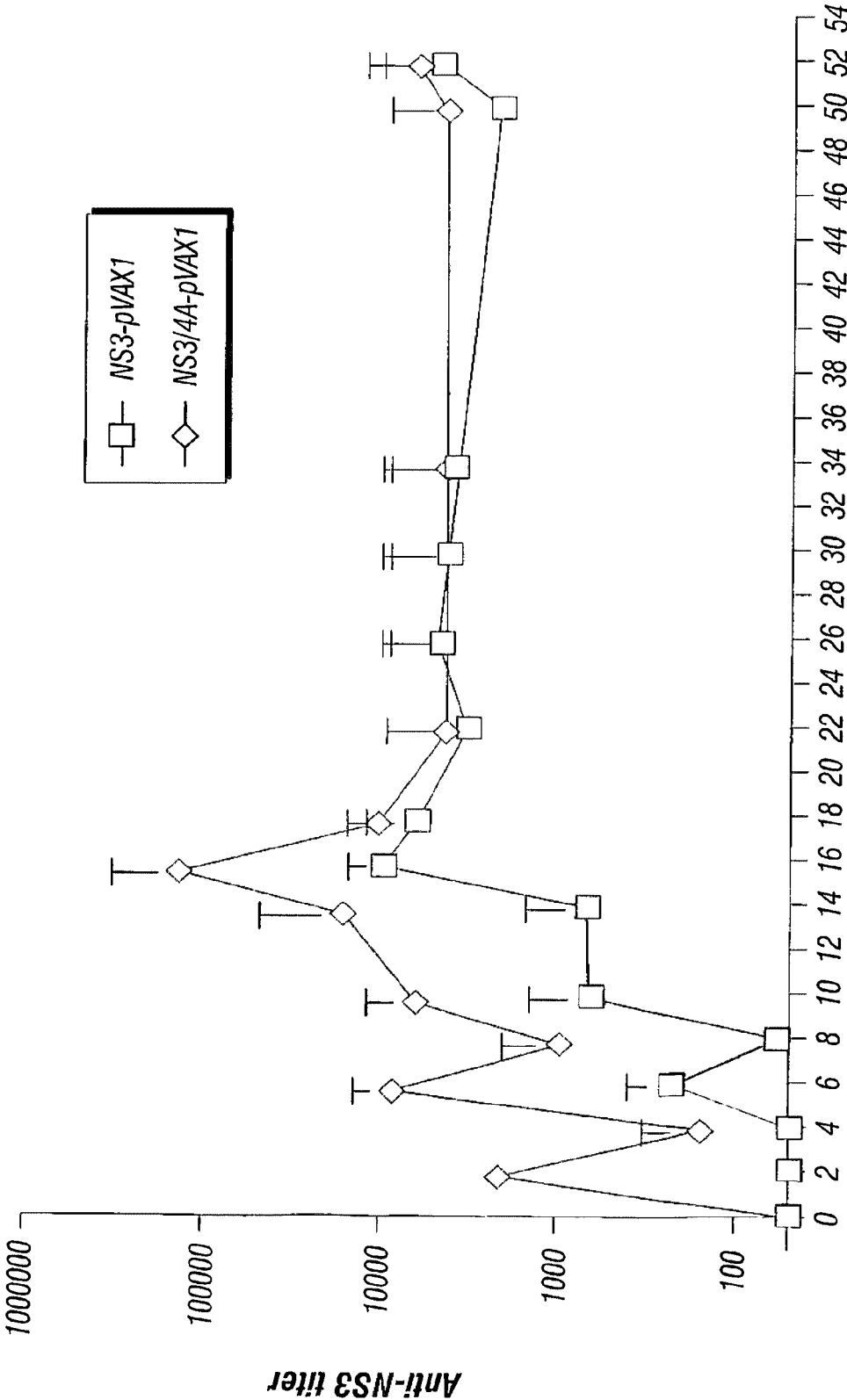


FIGURE 2

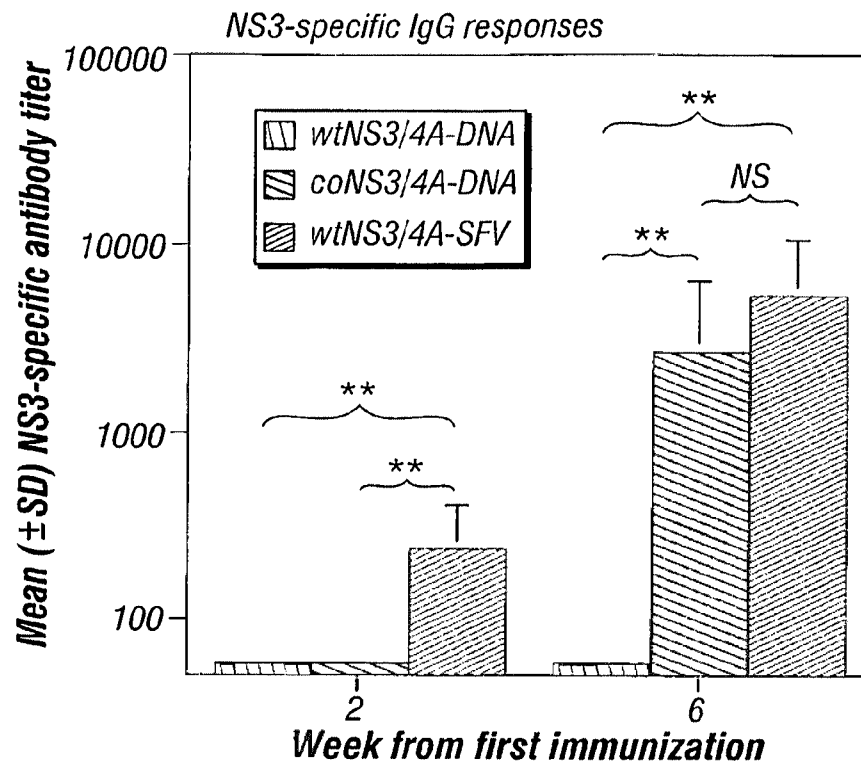


FIGURE 3A

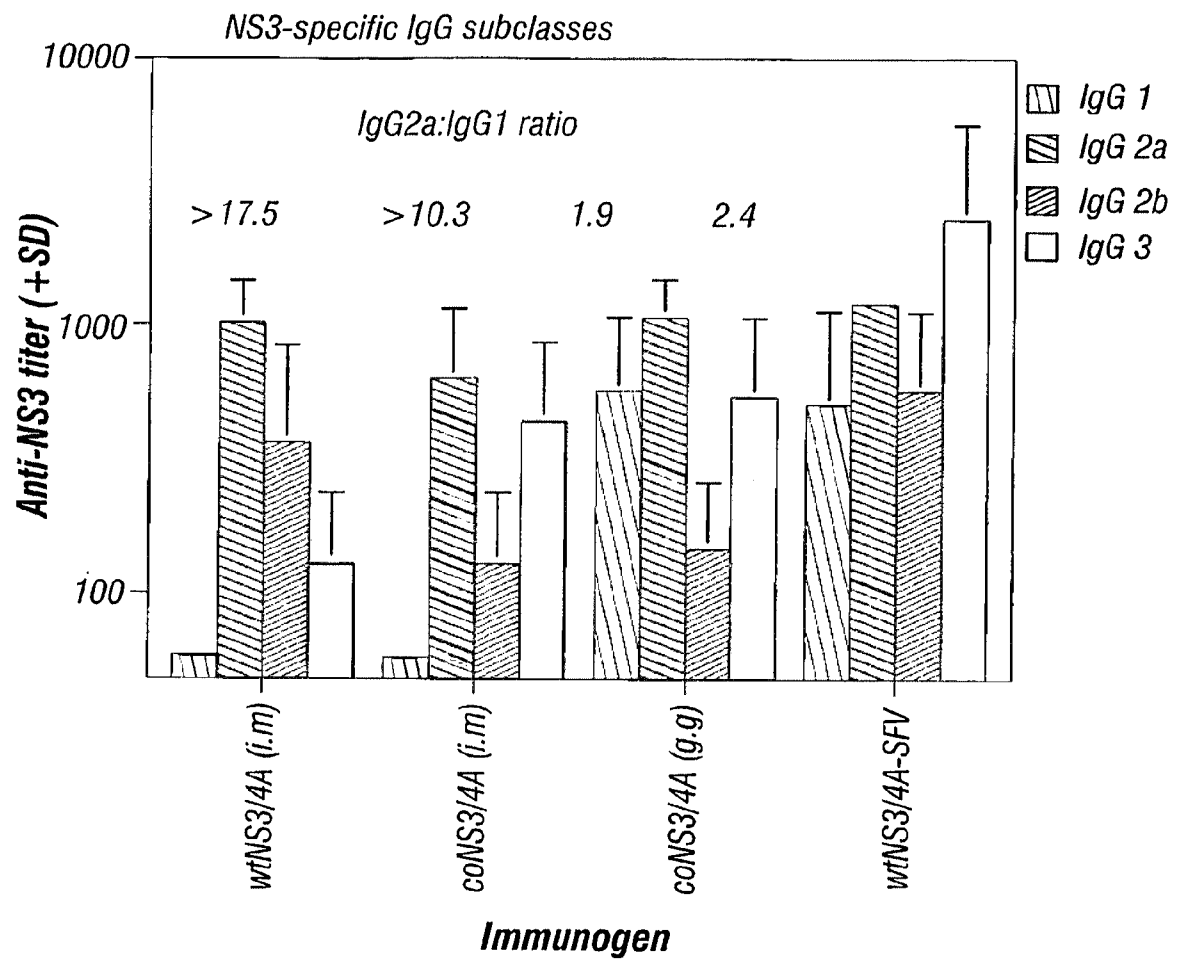
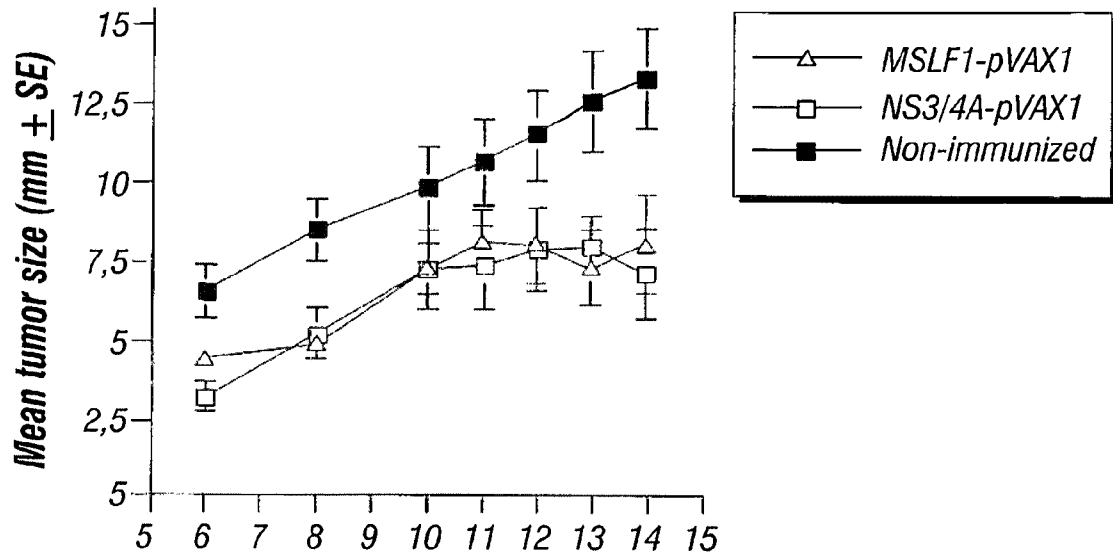


FIGURE 3B

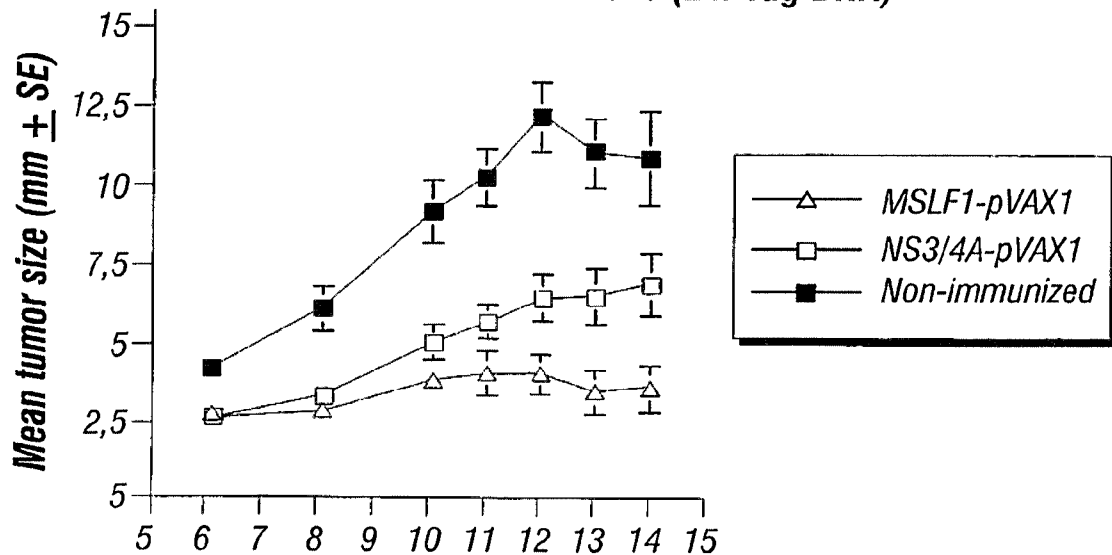
***In vivo protection against tumor growth after
one Gene Gun immunization (1 x 4ug DNA)***



Days from s.c. injection of 1×10^6 NS3/4A-Sp2/0 cells

FIGURE 4A

***In vivo protection against tumor growth after
two Gene Gun immunizations (2 x 4ug DNA)***



Days from s.c. injection of 1×10^6 NS3/4A-Sp2/0 cells

FIGURE 4B

***In vivo protection against tumor growth after
one Gene Gun immunization (3 x 4ug DNA)***

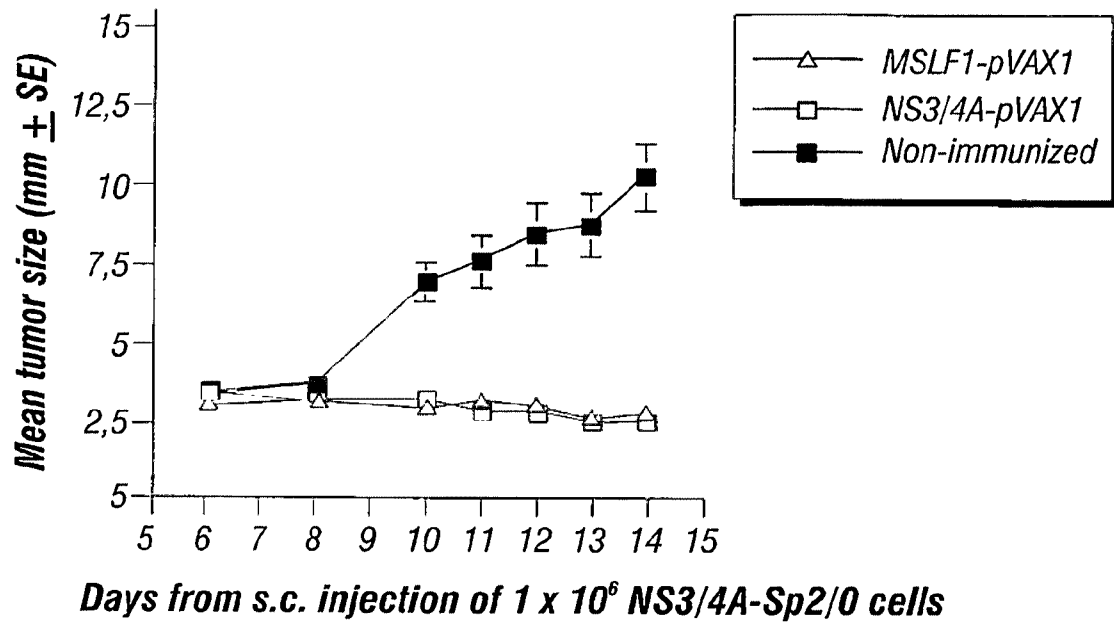


FIGURE 5

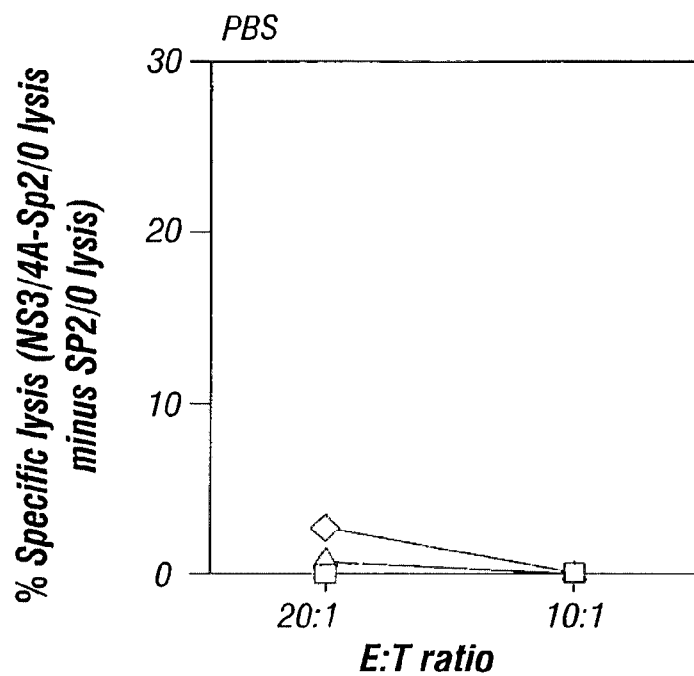


FIGURE 6A

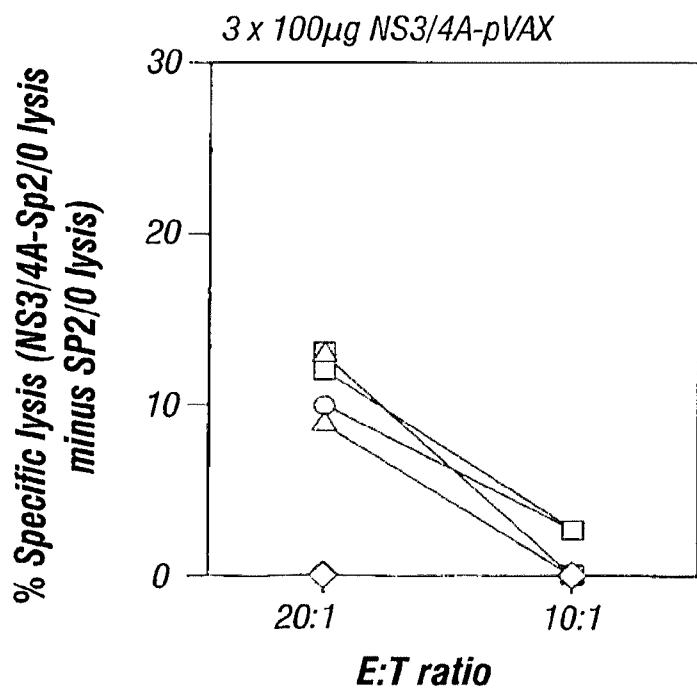


FIGURE 6B

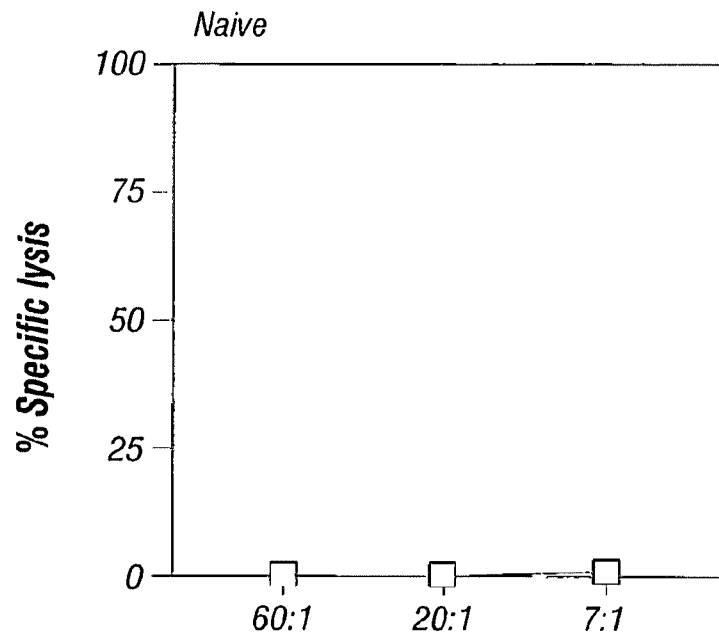


FIGURE 7A

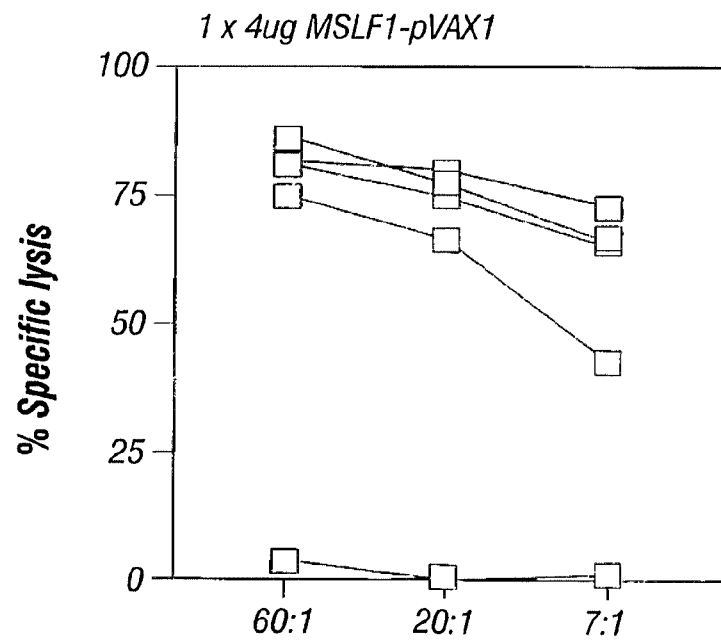


FIGURE 7B

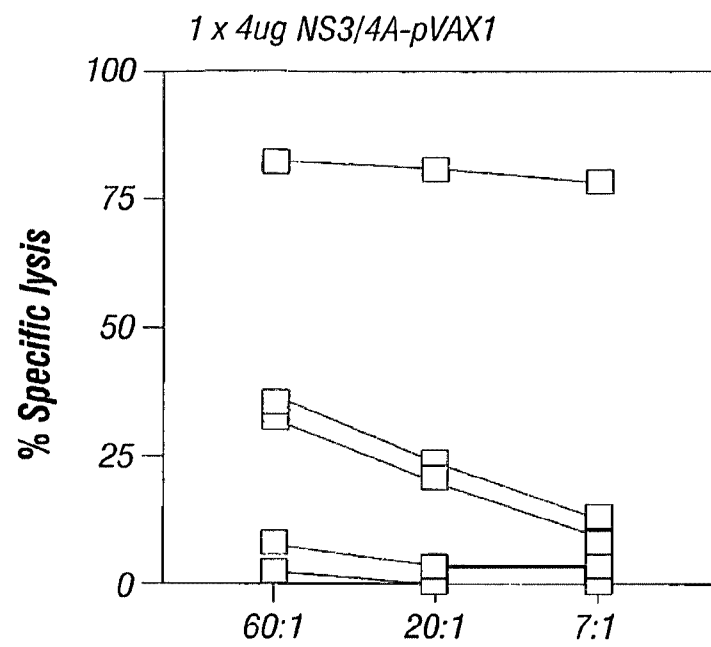


FIGURE 7C

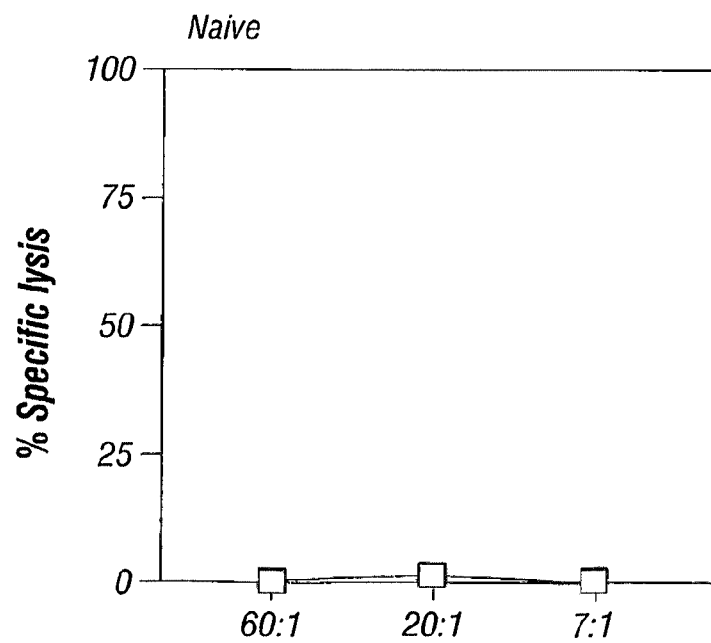


FIGURE 7D

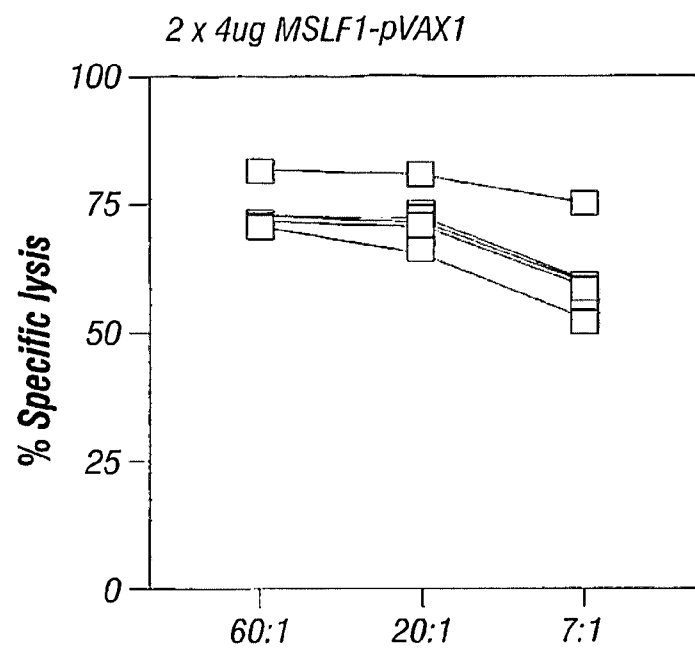


FIGURE 7E

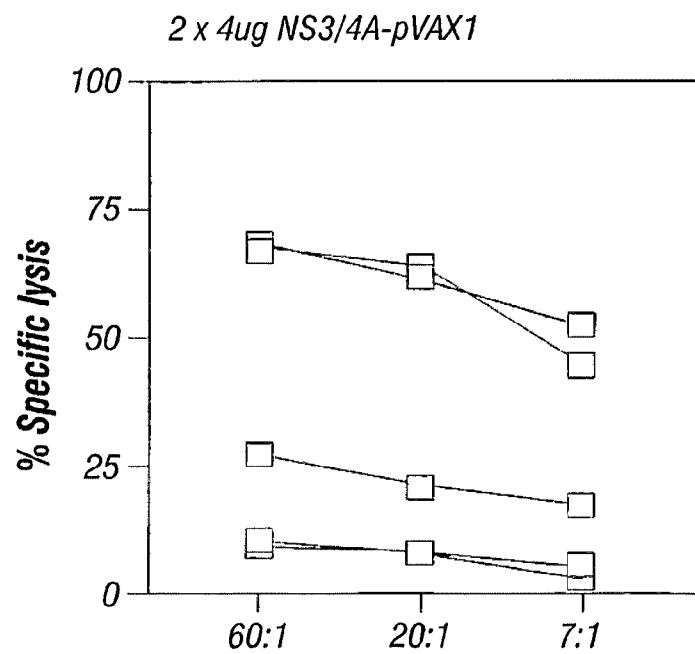


FIGURE 7F

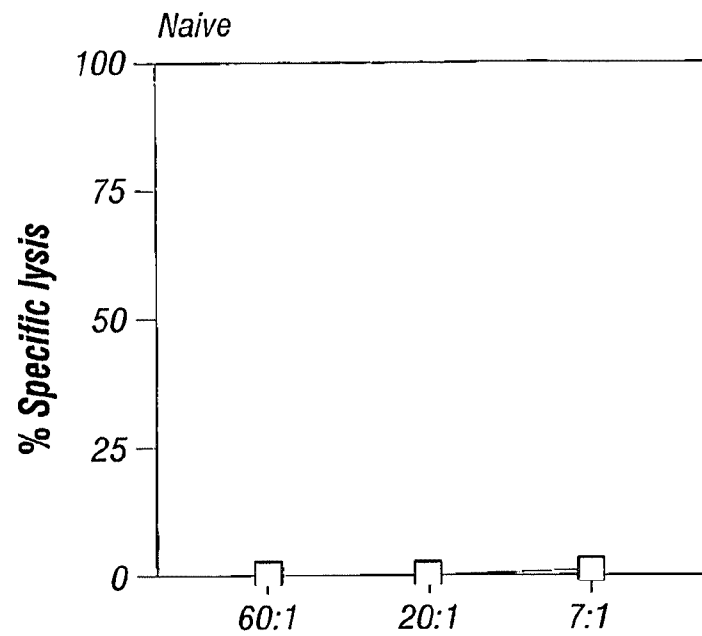


FIGURE 7G

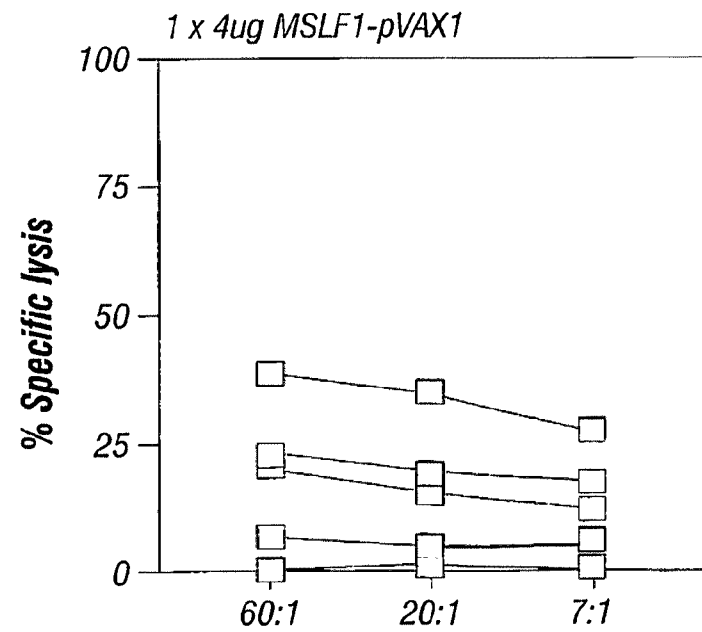


FIGURE 7H

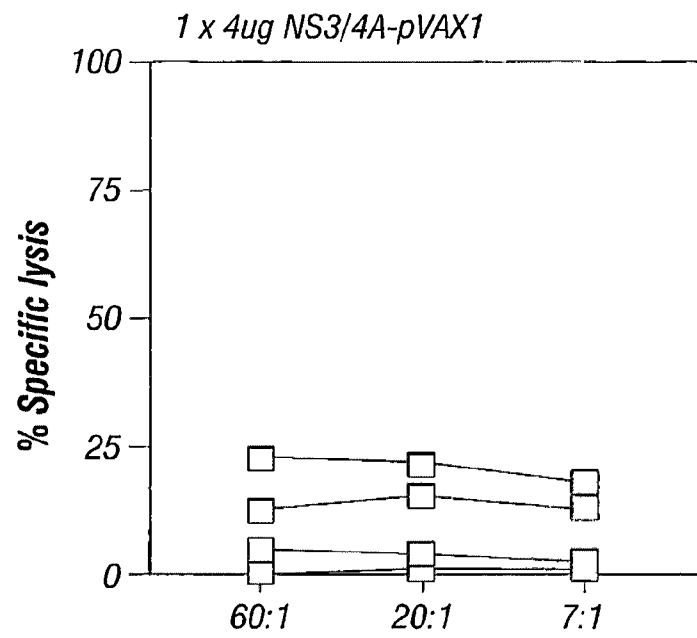


FIGURE 7I

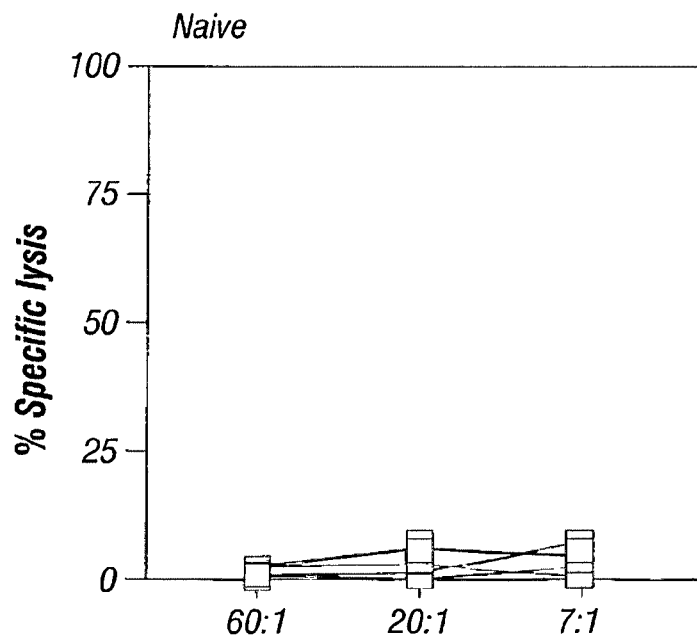


FIGURE 7J

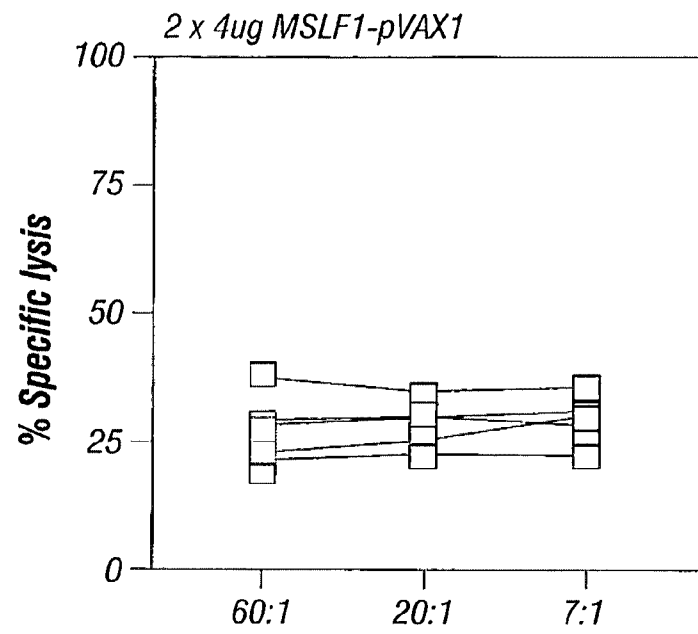


FIGURE 7K

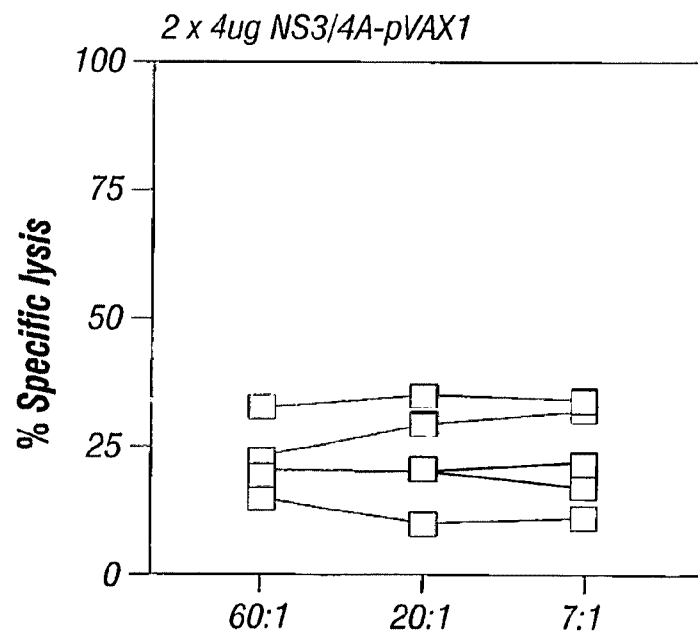


FIGURE 7L

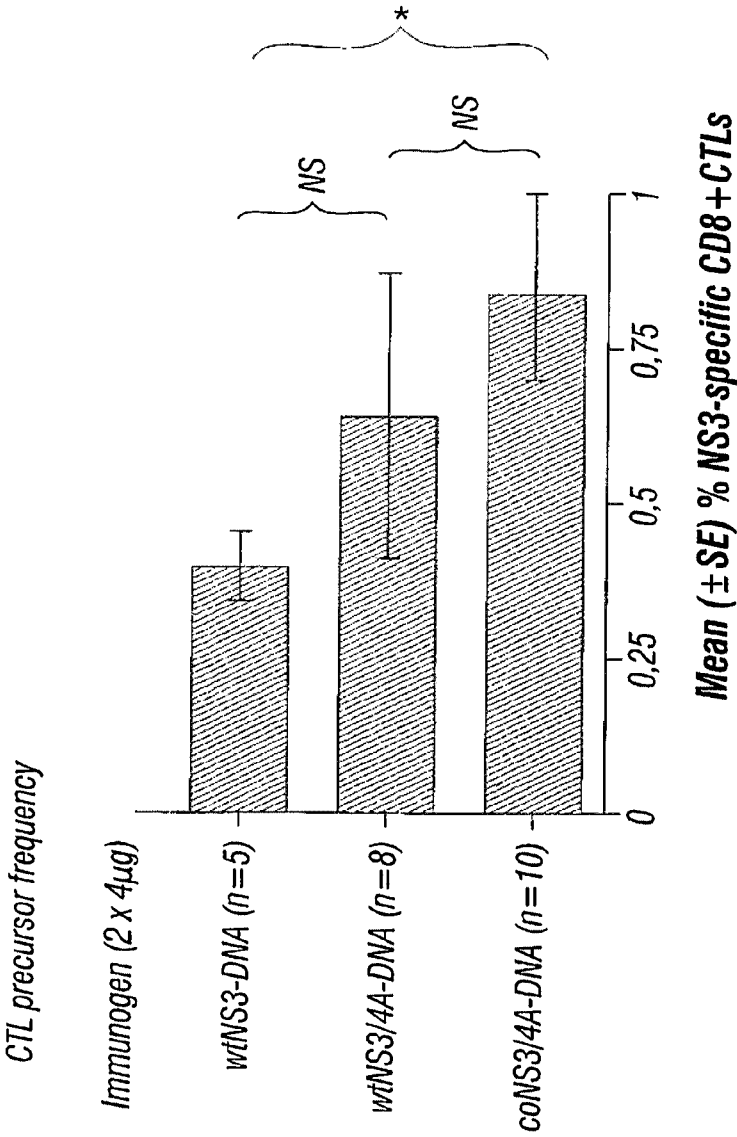


FIGURE 8A

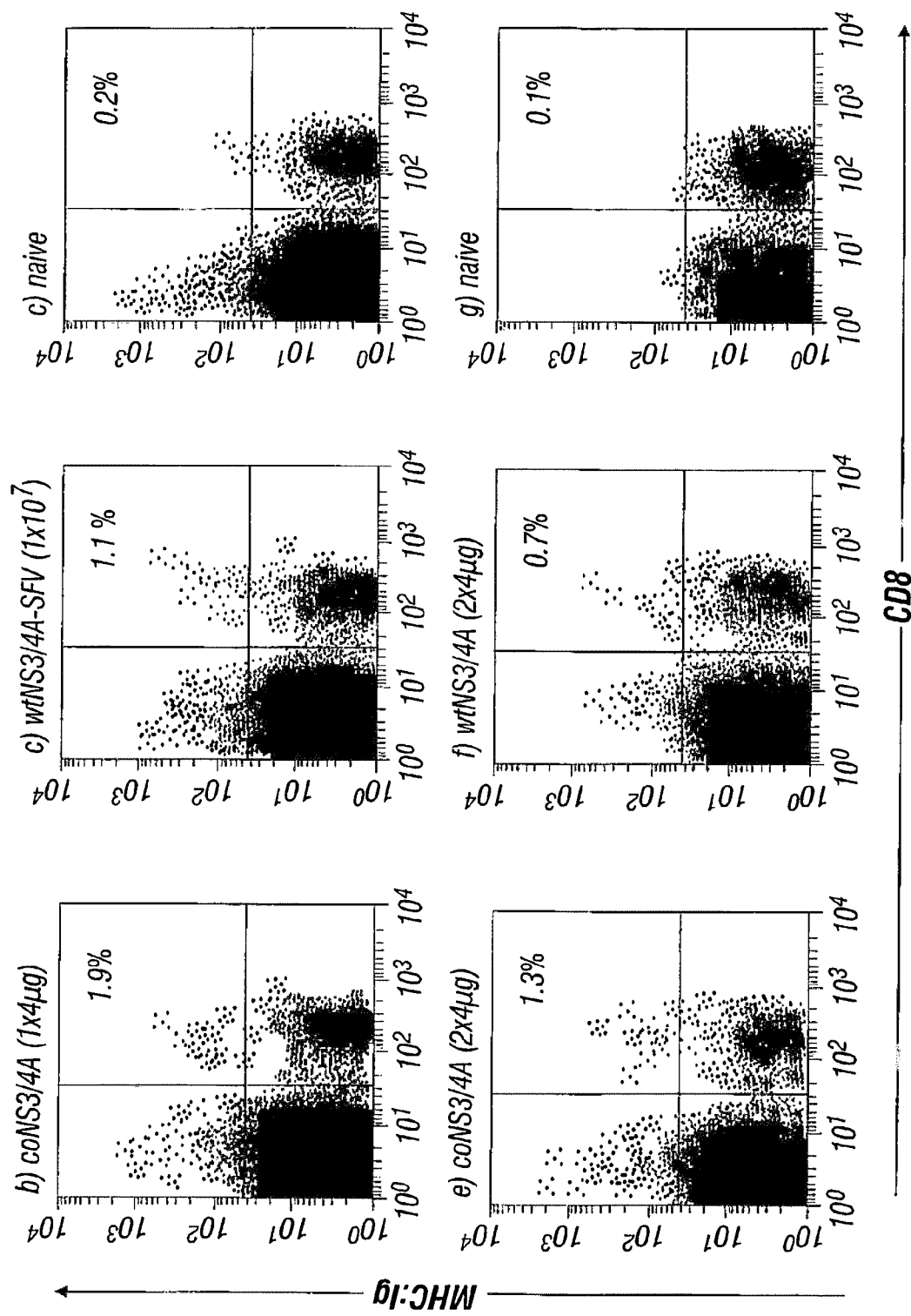


FIGURE 8B

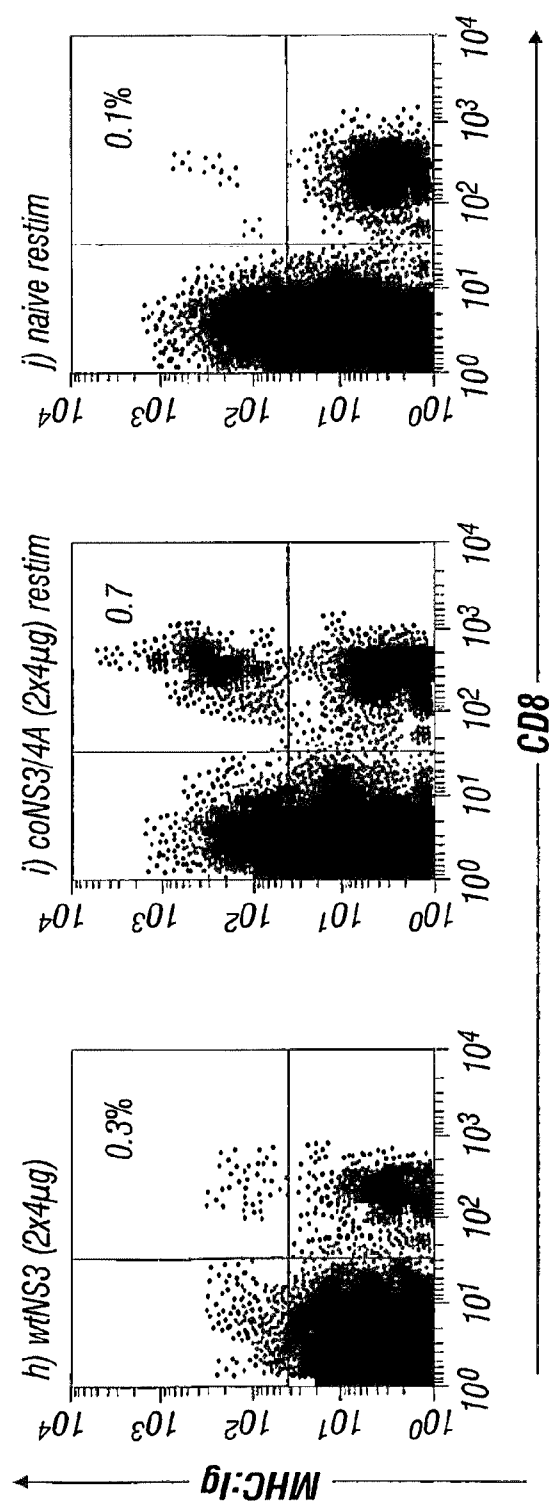


FIGURE 8B
(continued)

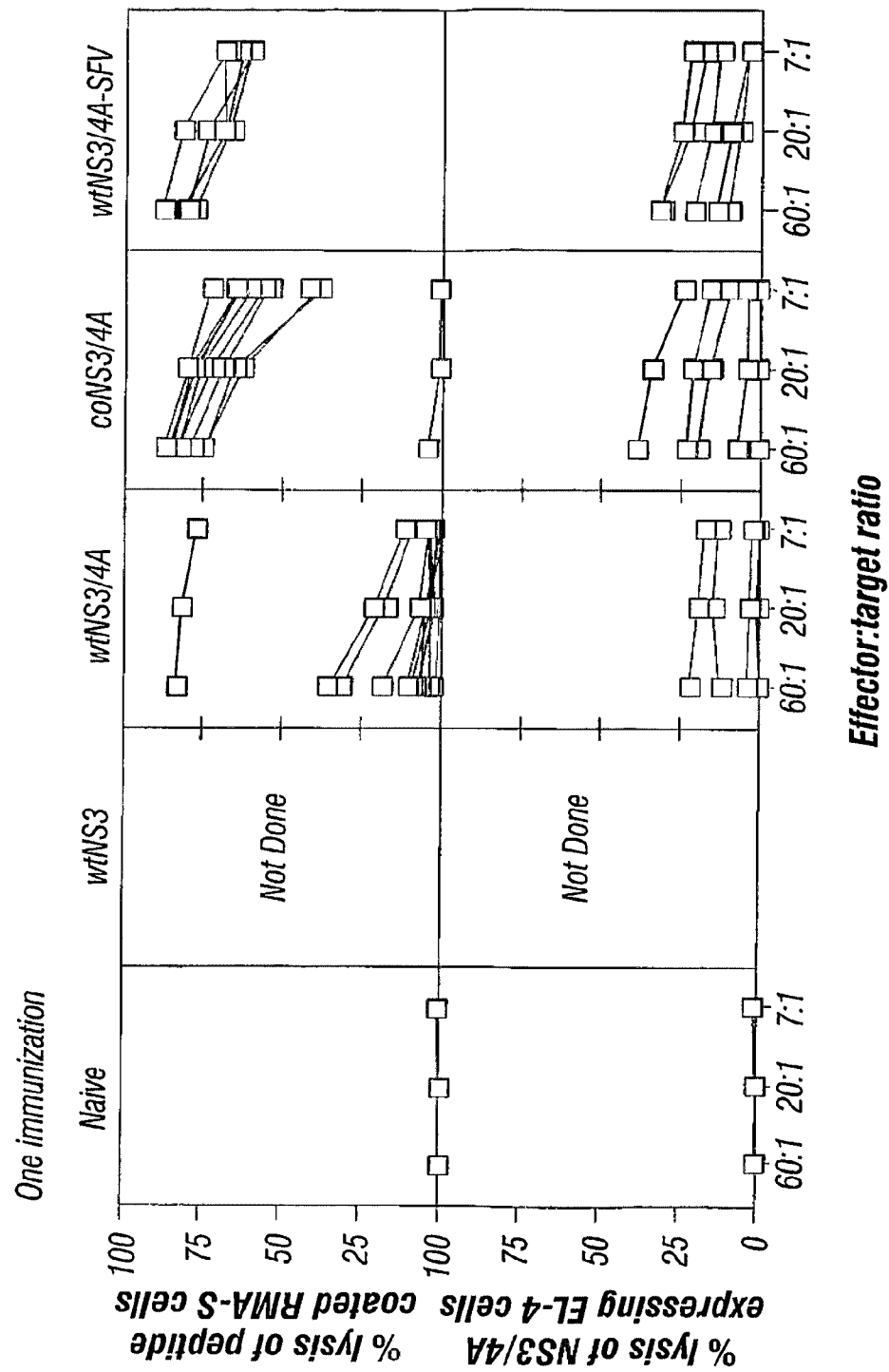


FIGURE 9A

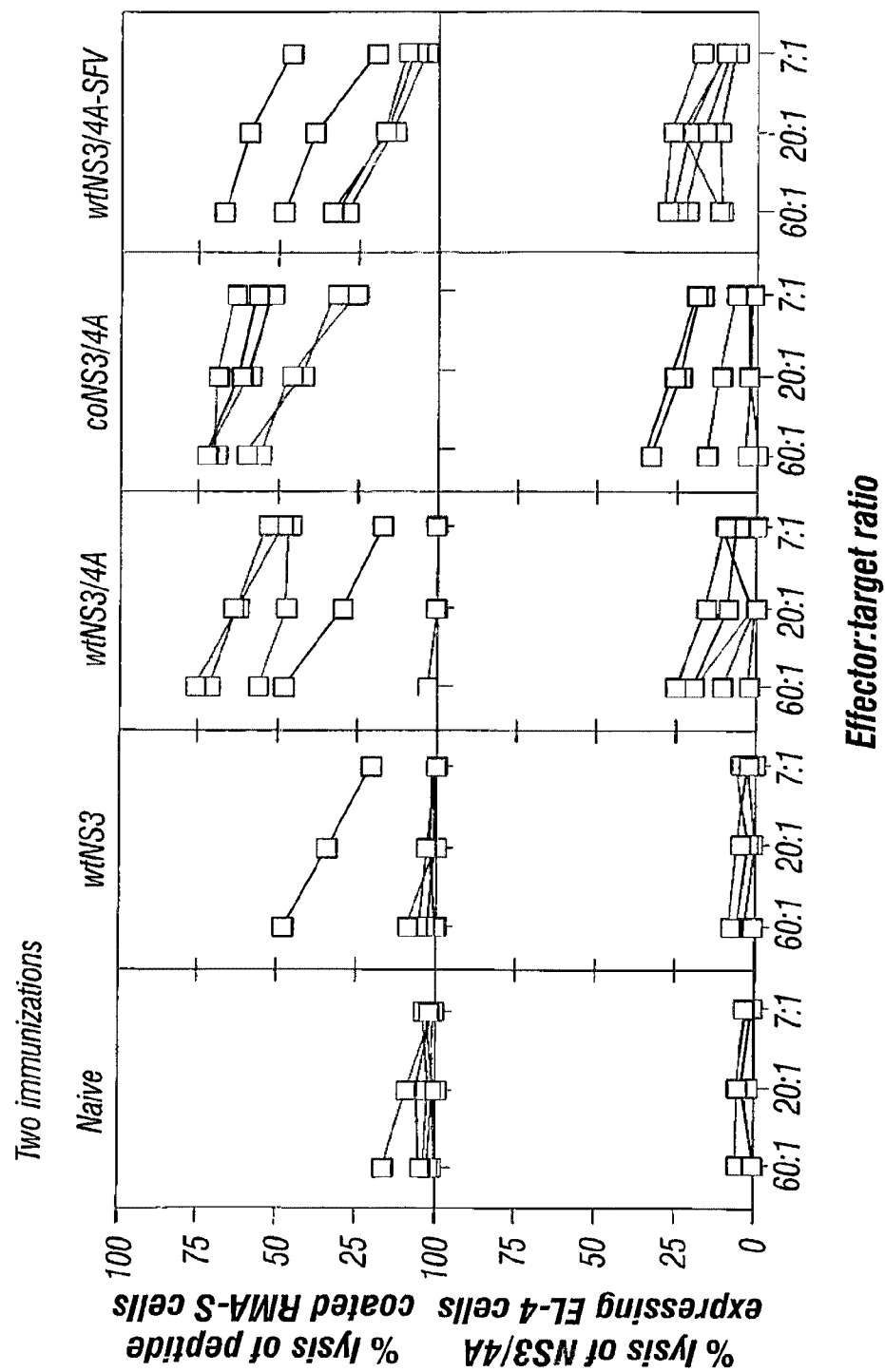


FIGURE 9B

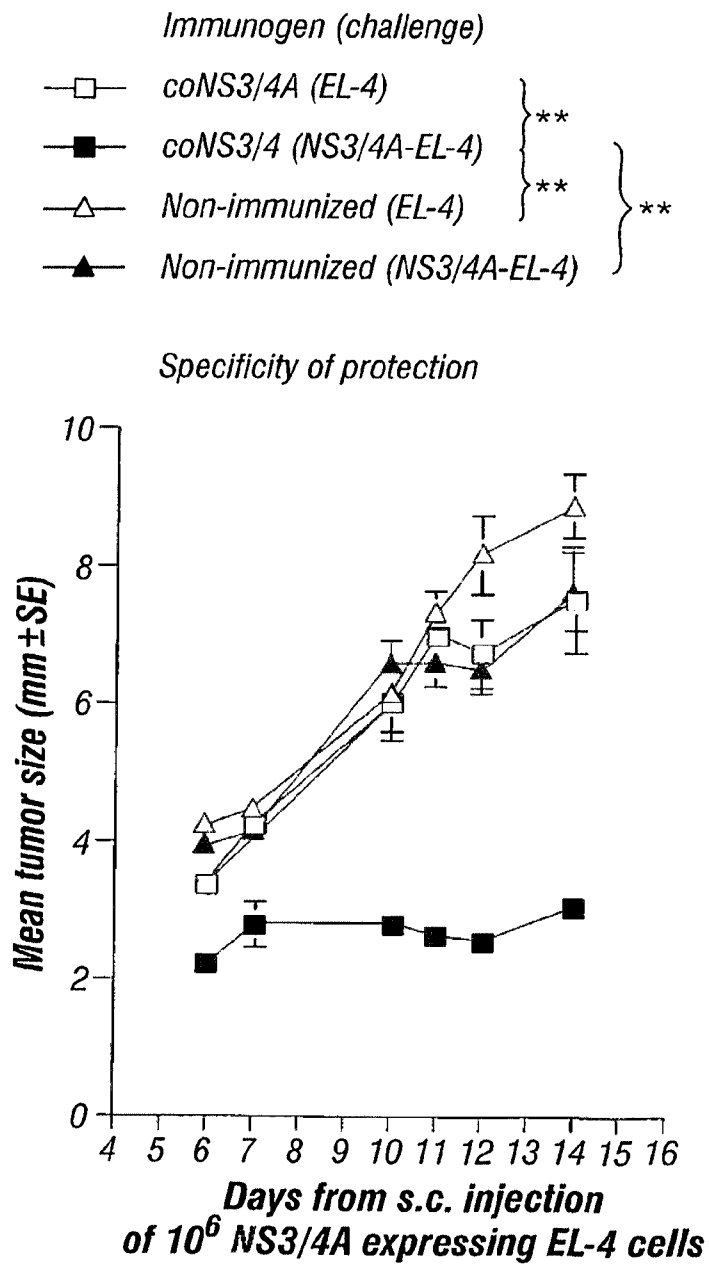


FIGURE 10A

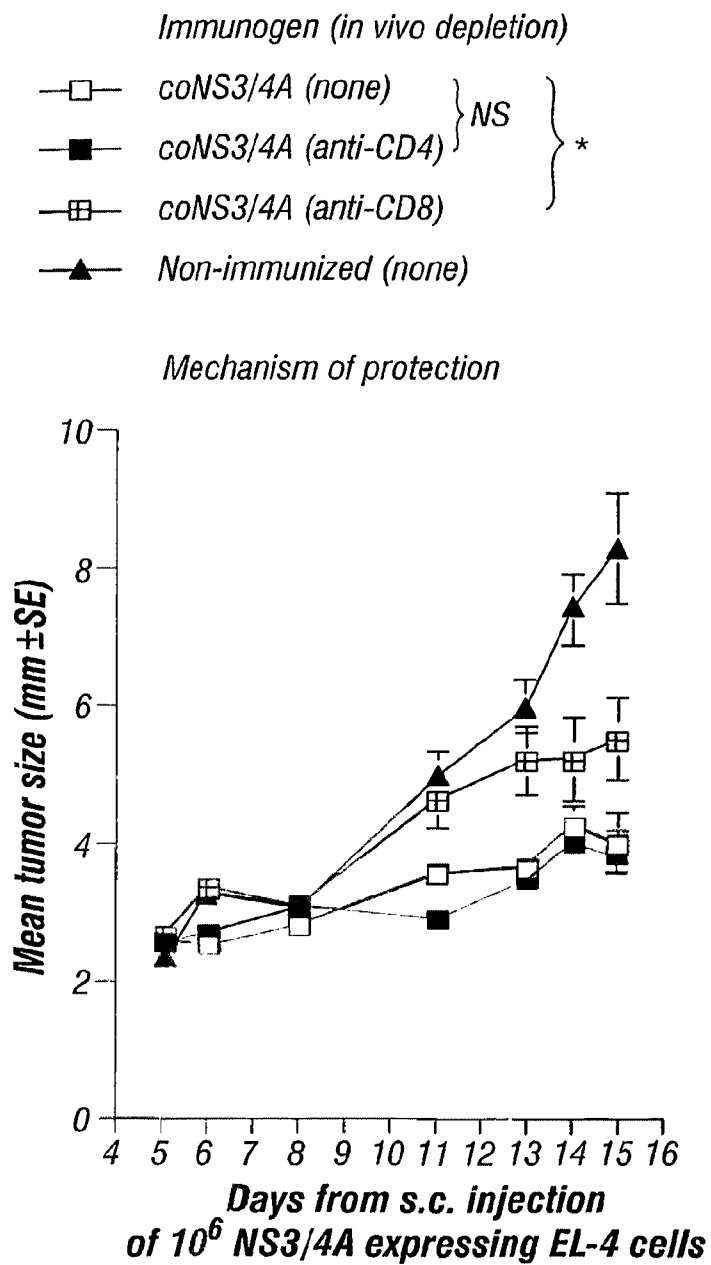
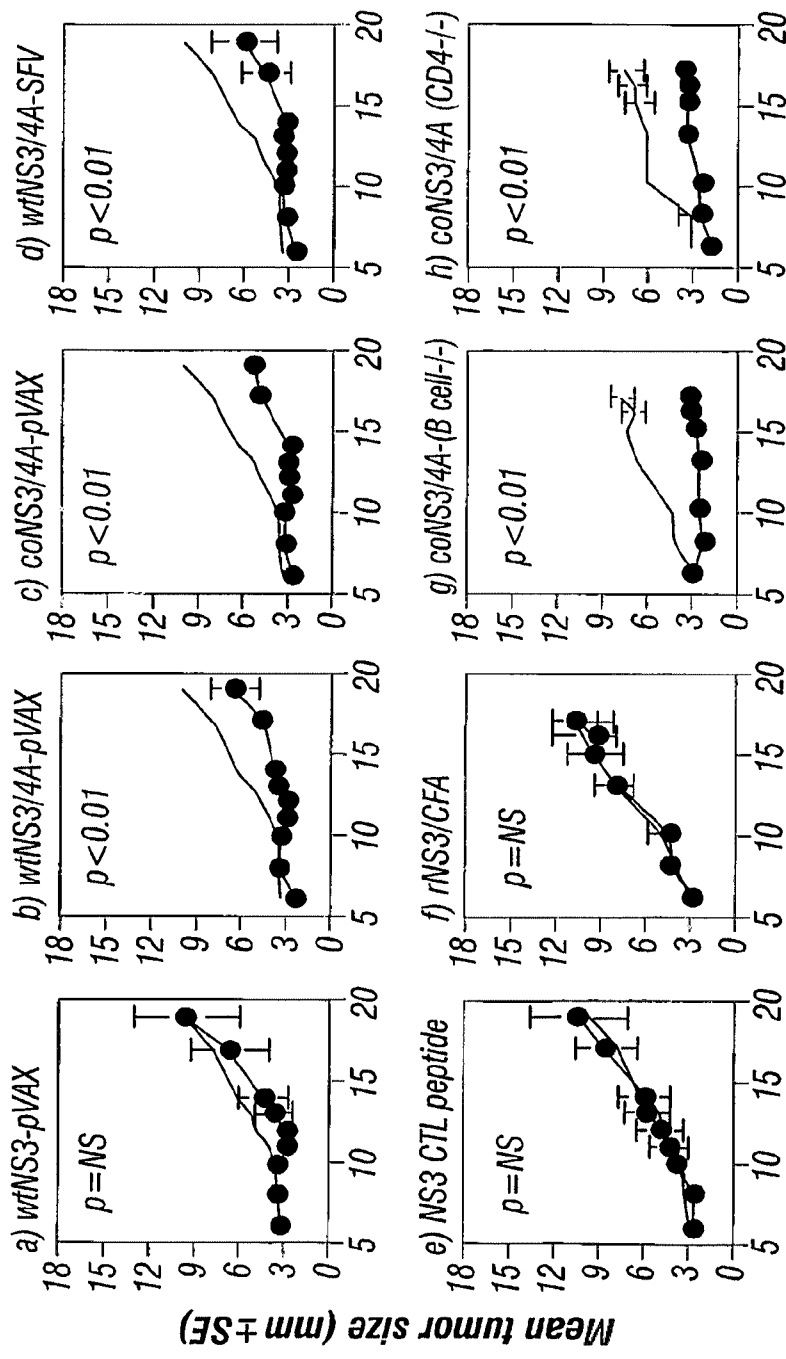


FIGURE 10B



Days from s.c. injection of 10^6 NS3/4A - expressing EL-4 cells

FIGURE 11

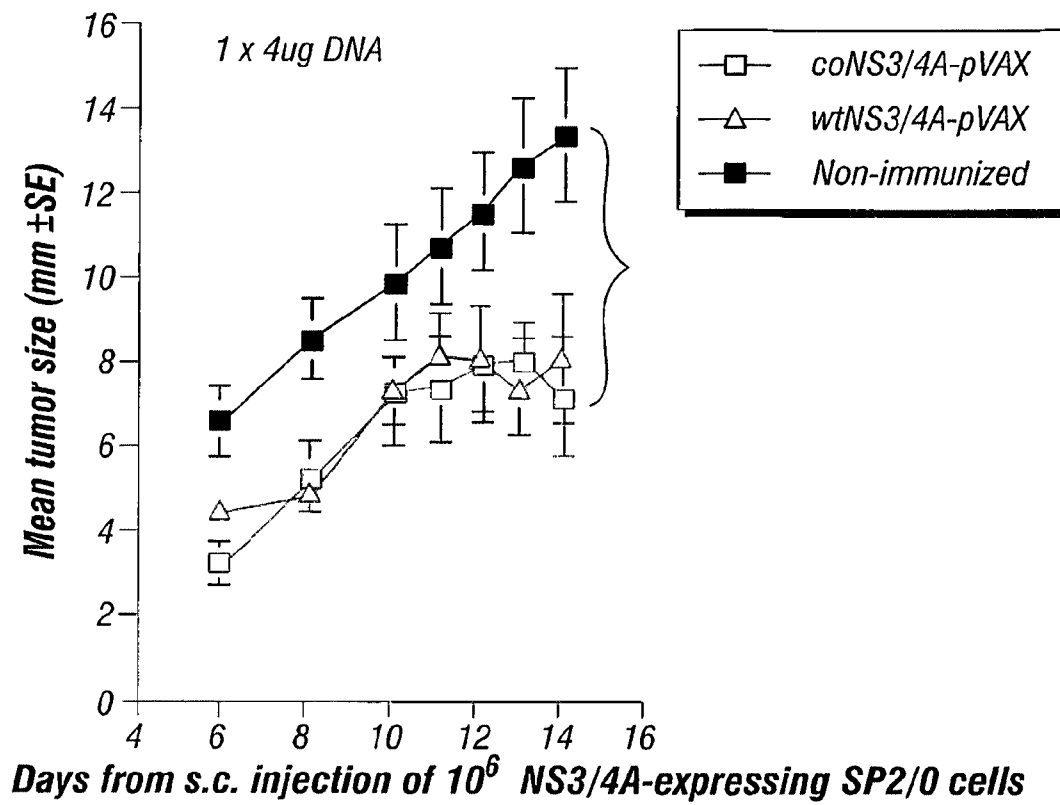


FIGURE 12A

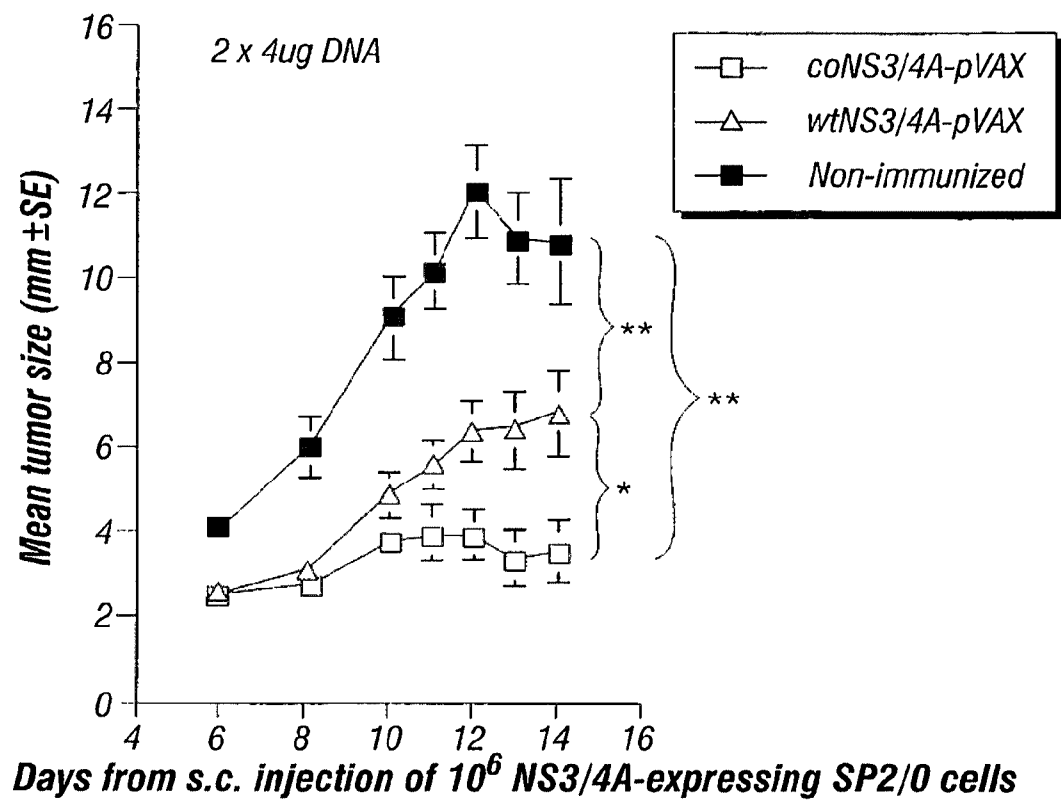


FIGURE 12B

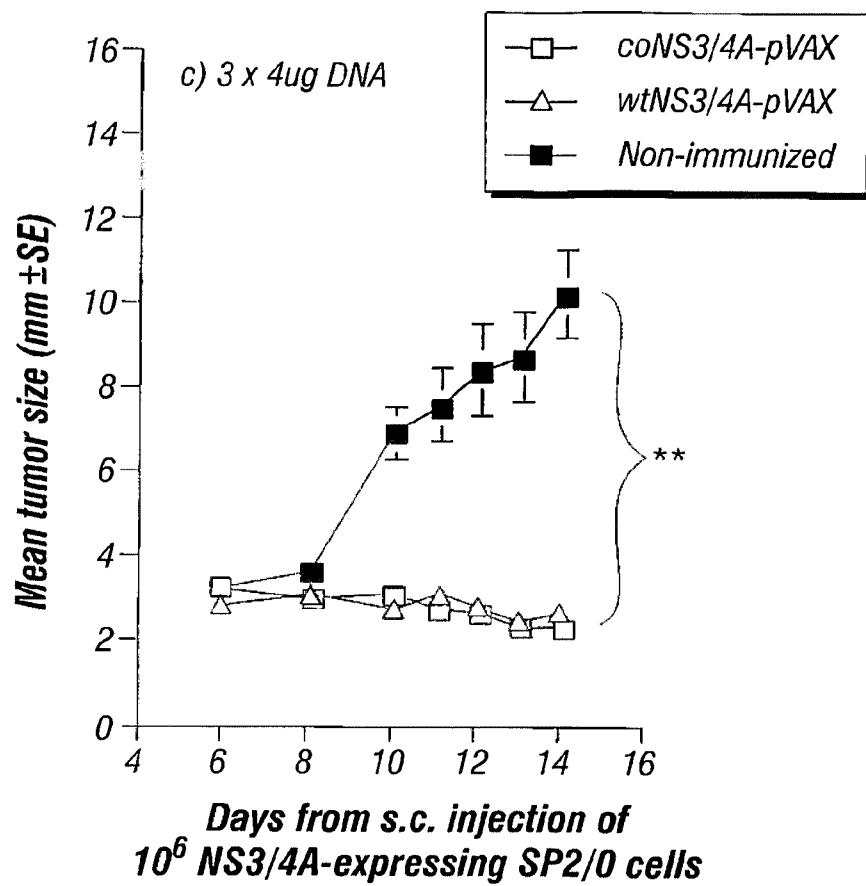


FIGURE 12C

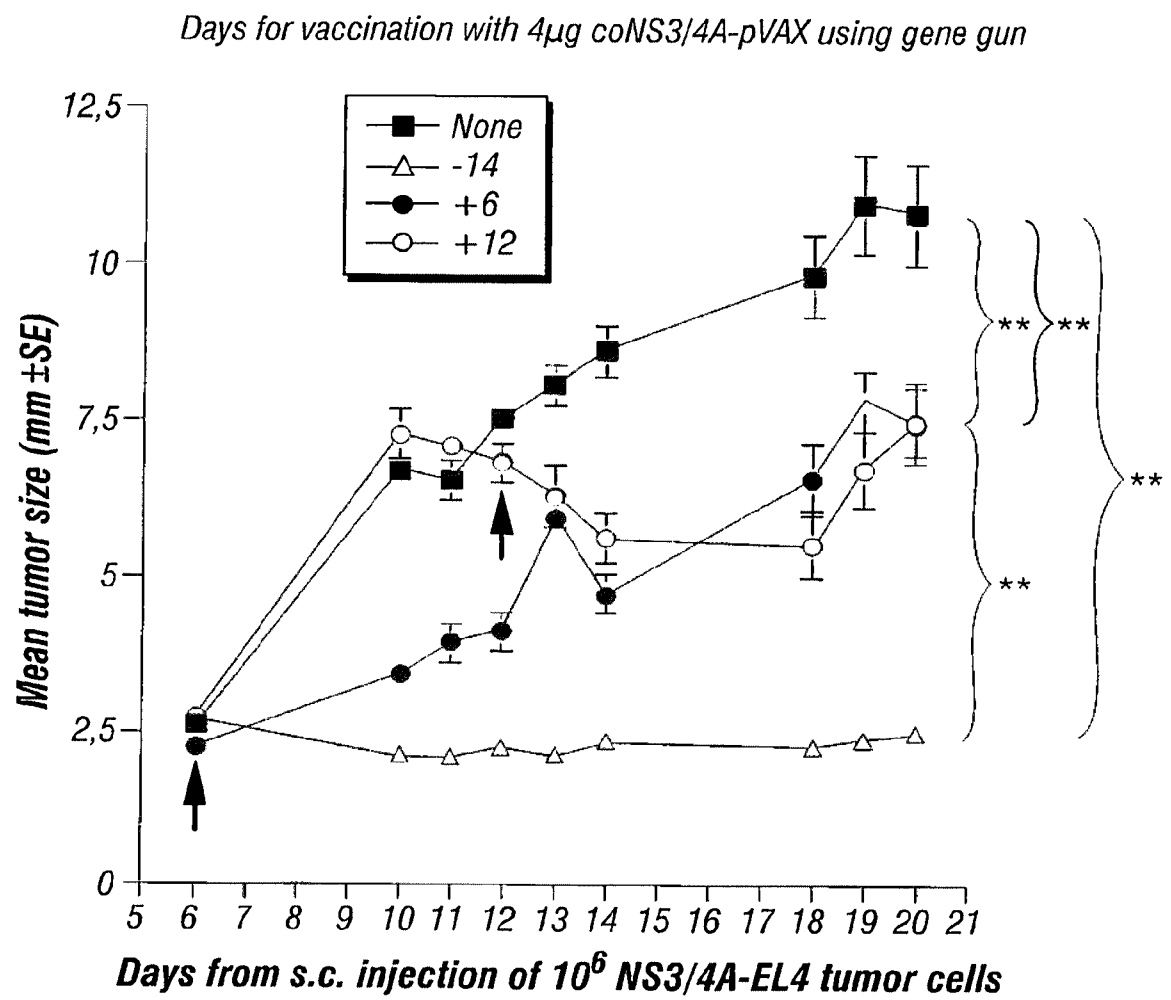


FIGURE 13

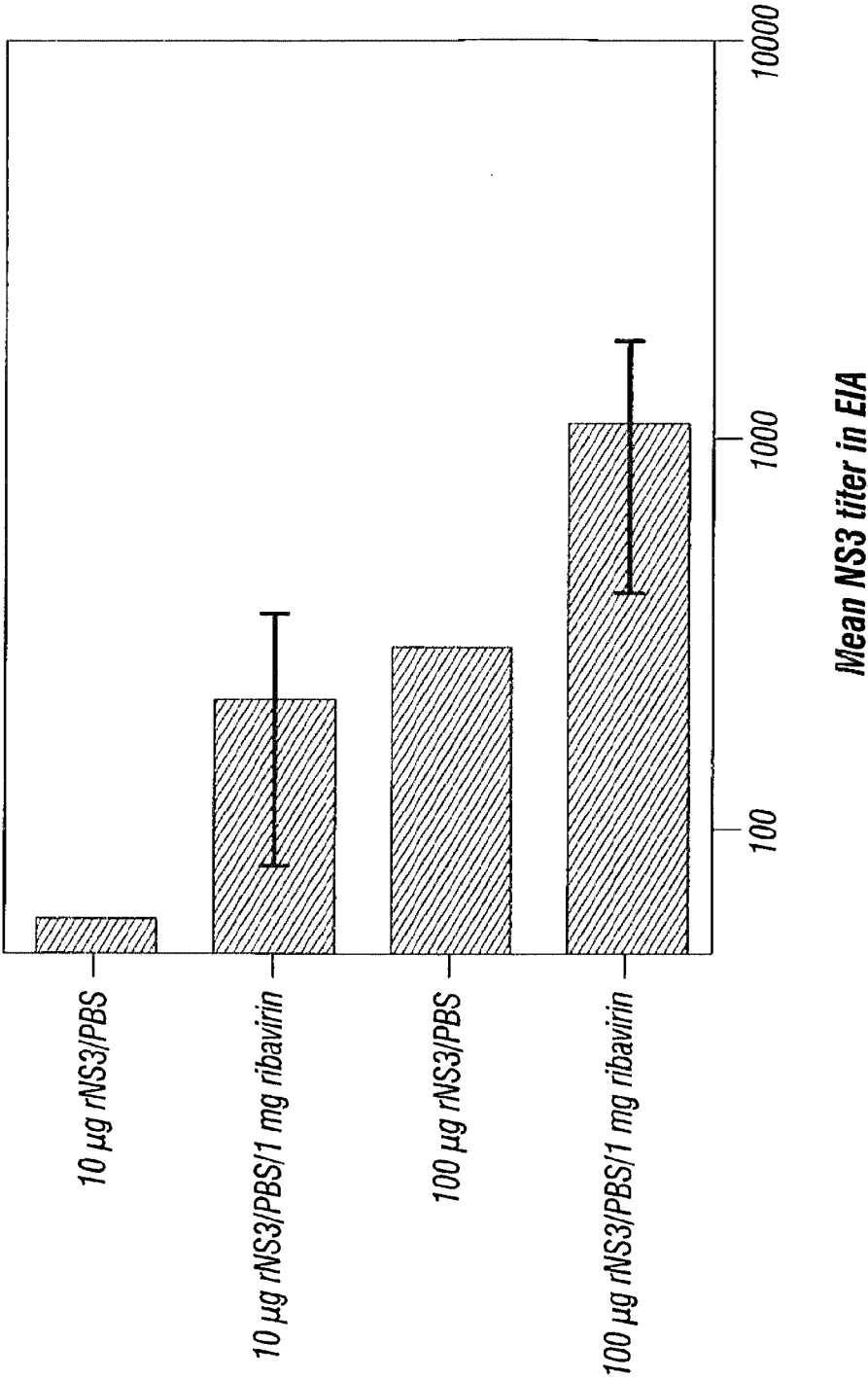


FIGURE 14

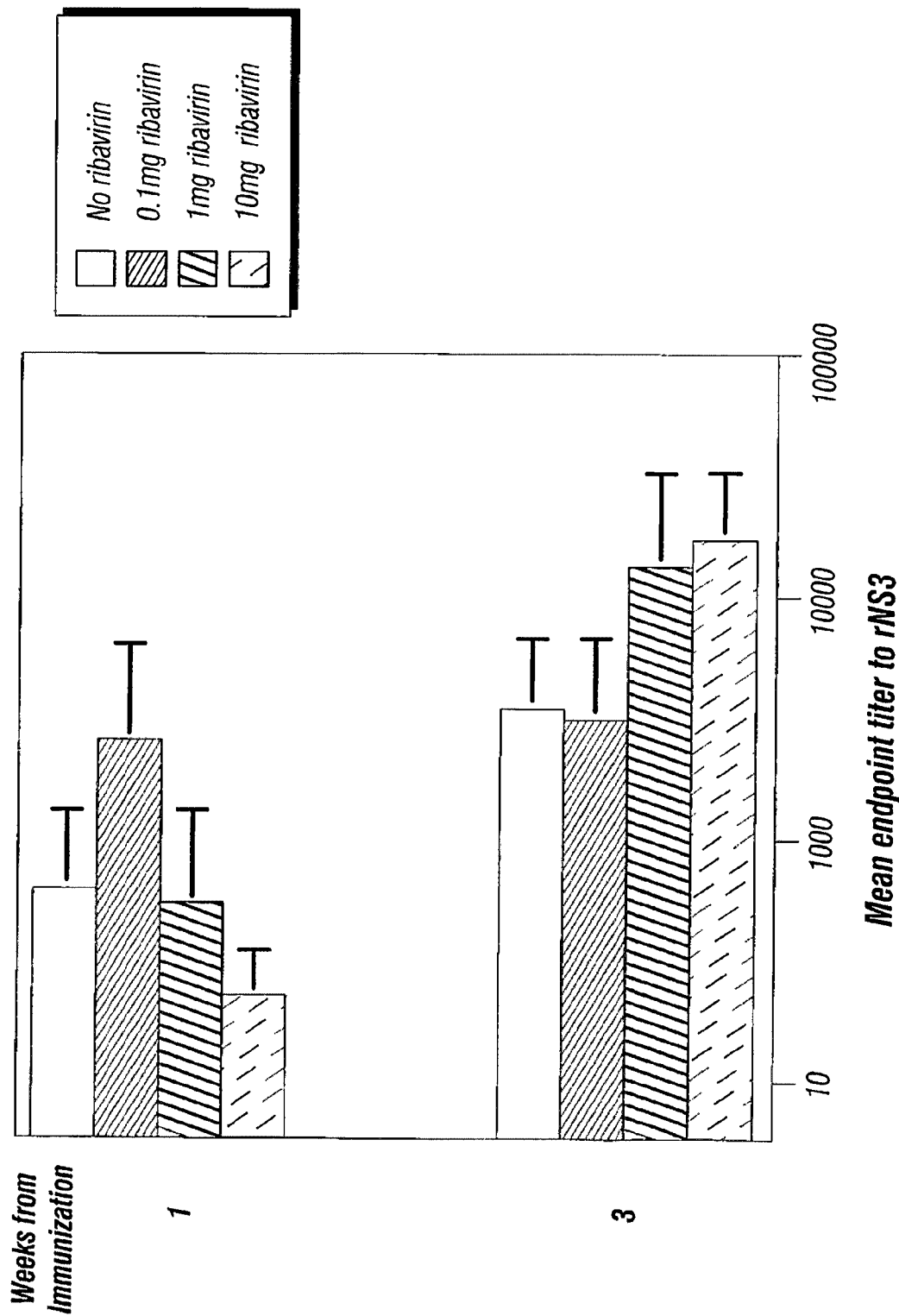


FIGURE 15

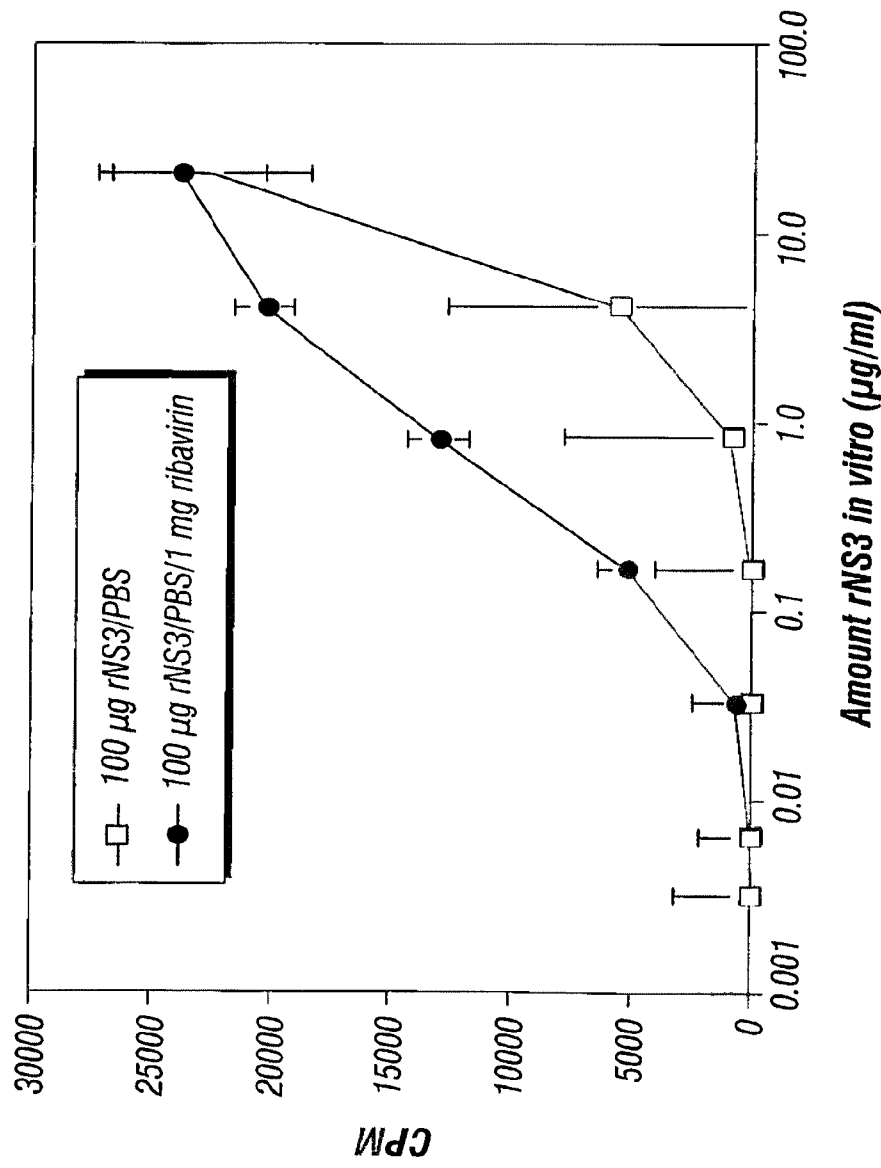


FIGURE 16

0 = Complete destruction of the protease activity



3 = NS3 amount equal to NS3-4A amount



6 = Total cleavage

APLTAYACQIRGLLGCIITSITGRDKNQVEGEVOIVS
 TAAQTRHATCINQVQWIVYHAGTRILASPKGPVIOM
 YTNVDQDLVGWPAPOGARSTTPCTCGSSDLYLVTRHA
 LVILPVRRRGQGRGSLSPRPISYLLKGSSGGFLLCRAE
 HAVGIERAANCTRGVAKAVDFIIVESLETIMRS

FIG. 17

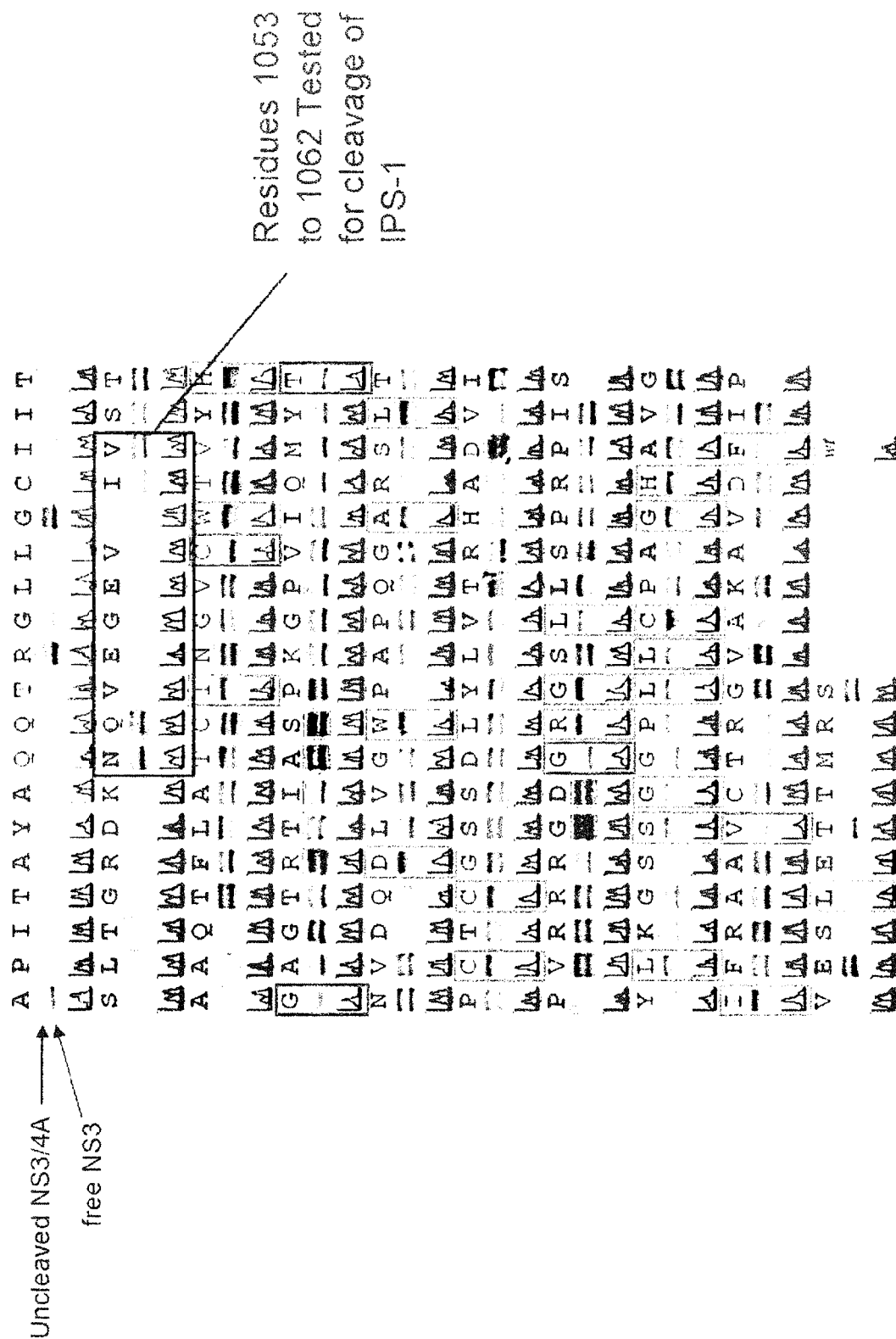


Figure 18

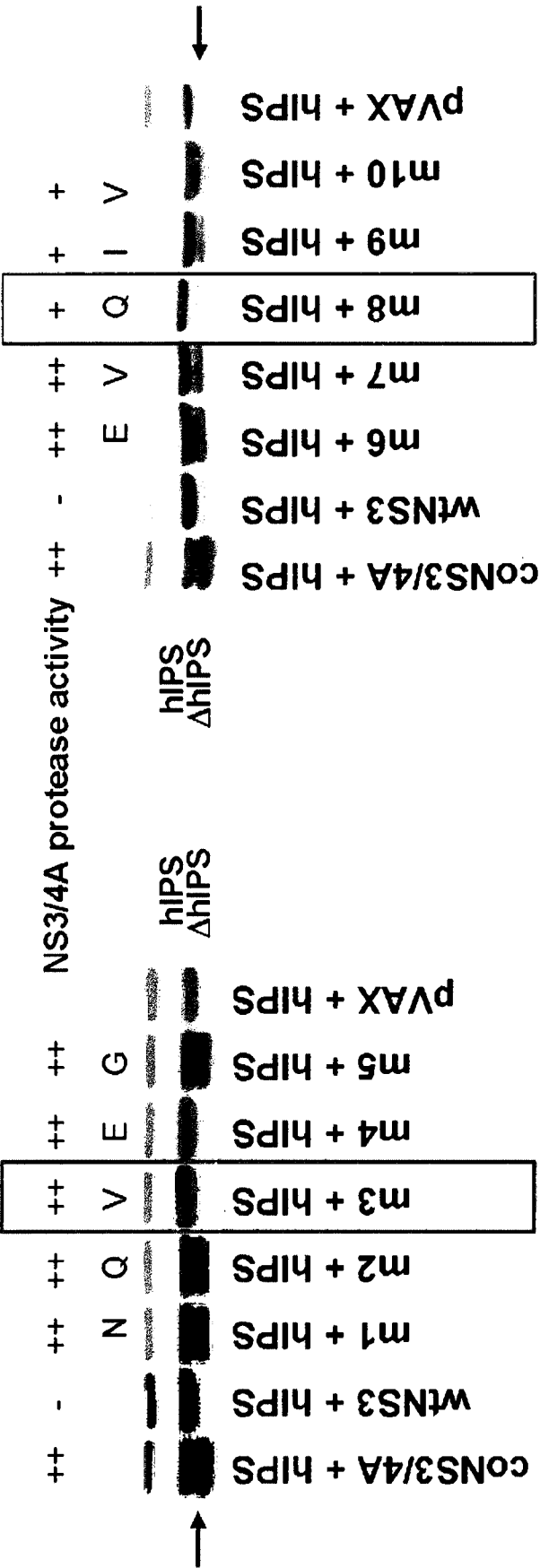


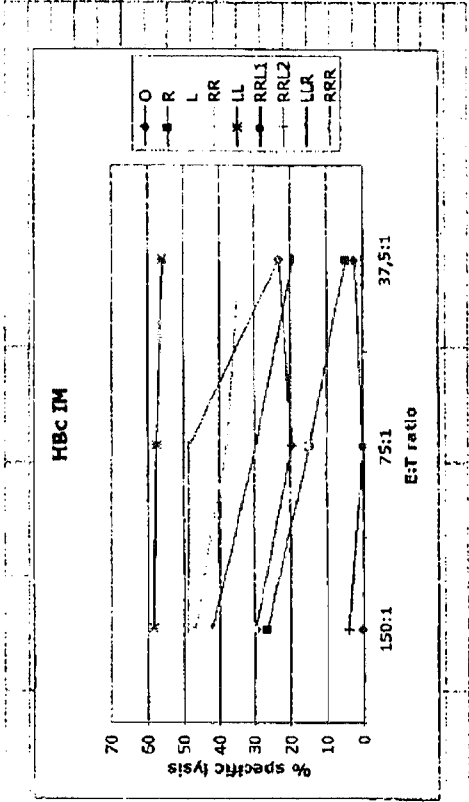
Figure 19A

Figure 19B

FIGURE 20A

— *i.m.* immunization

One immunization



2 x IM HBC

Two immunizations

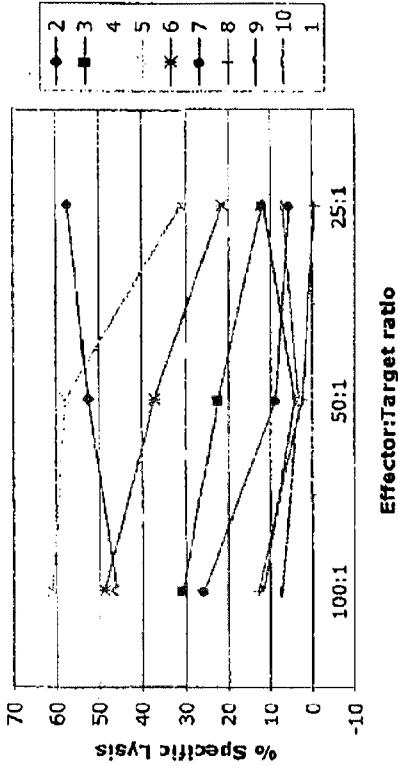
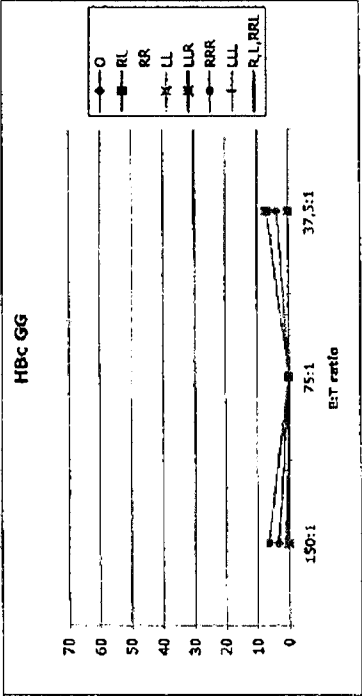
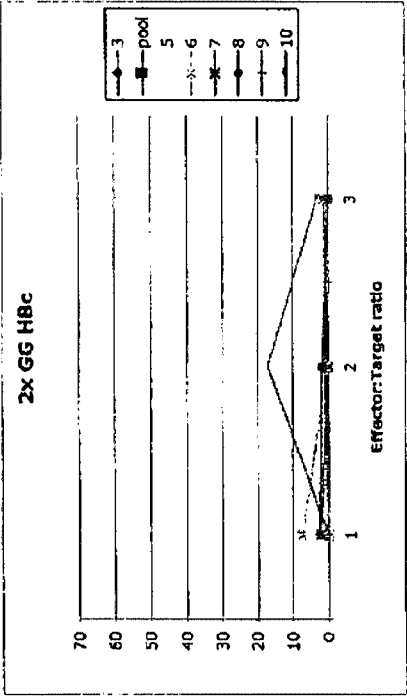


FIGURE 20B
- Gene gun
immunization



One immunization



Two immunizations

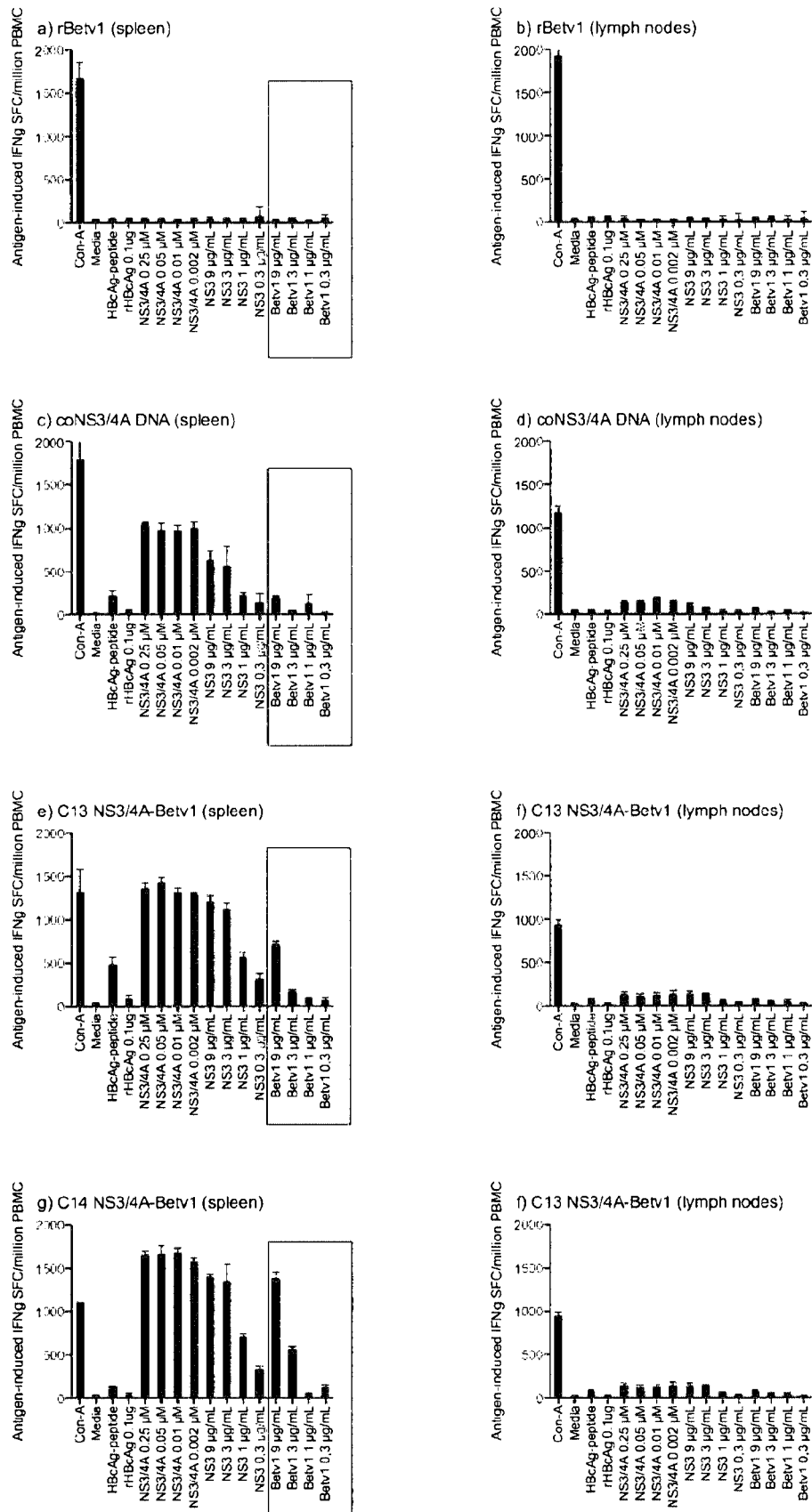


FIGURE 22

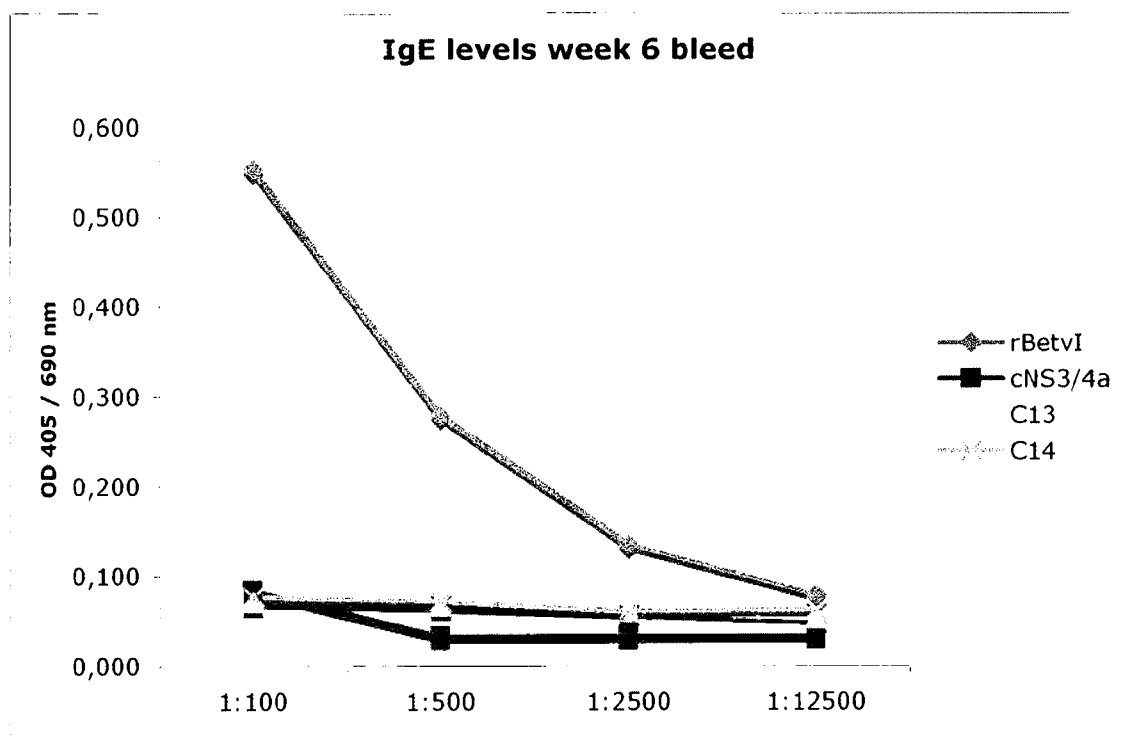


FIGURE 23

Detection of HBc specific lysis

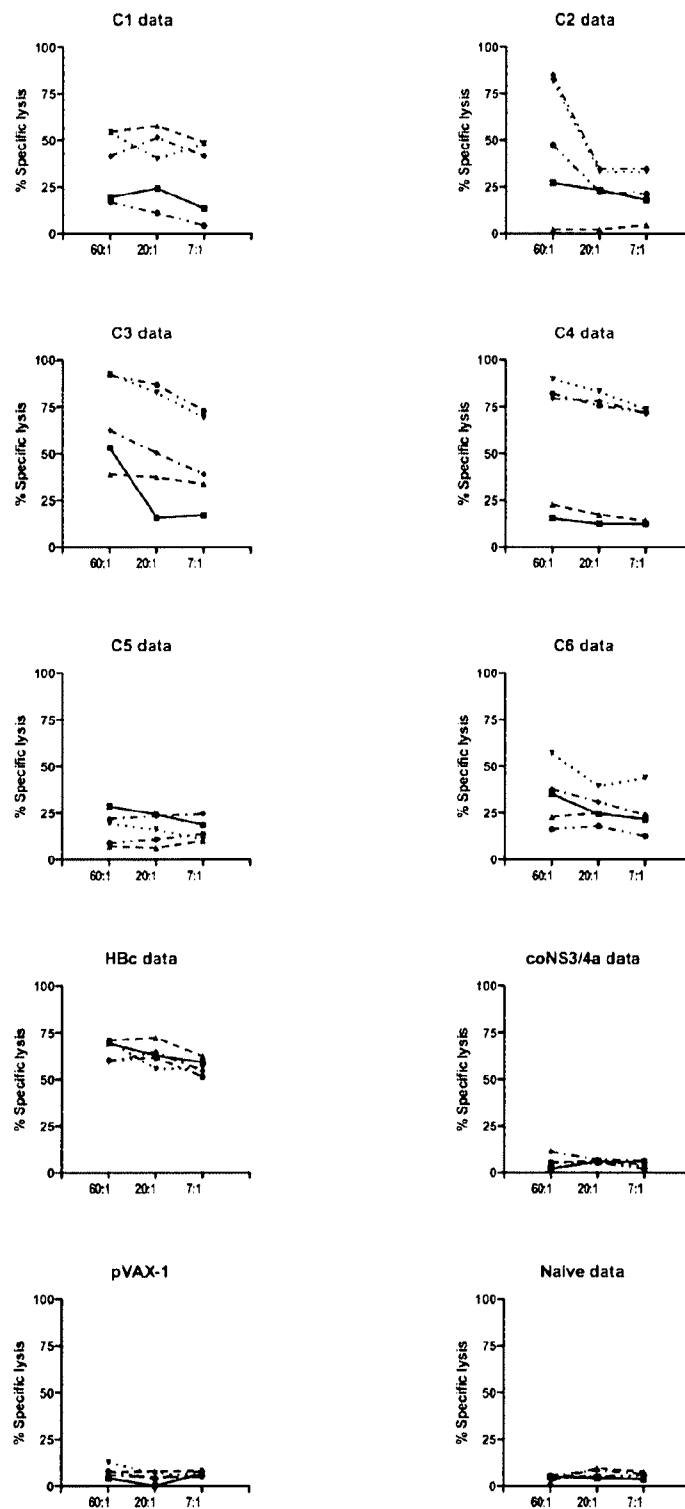


FIGURE 24

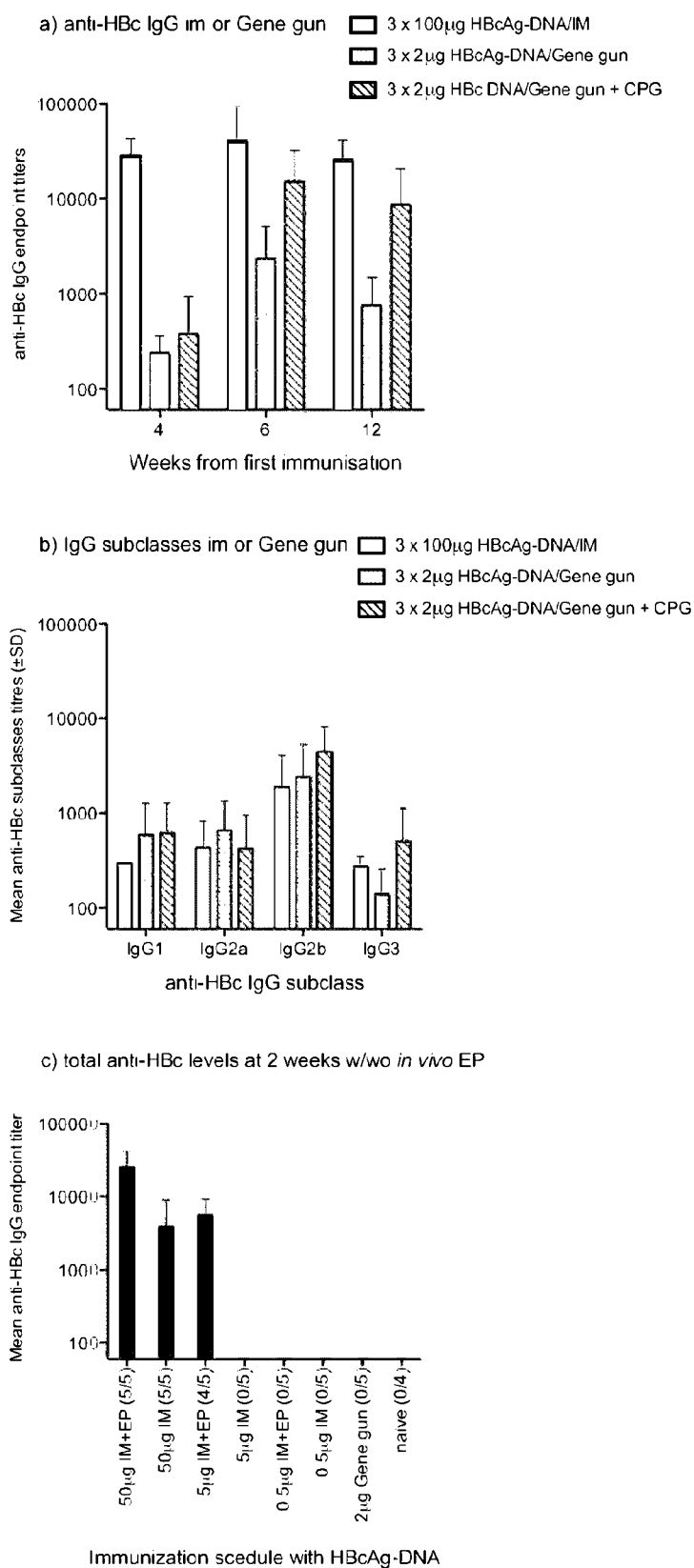


Figure 25

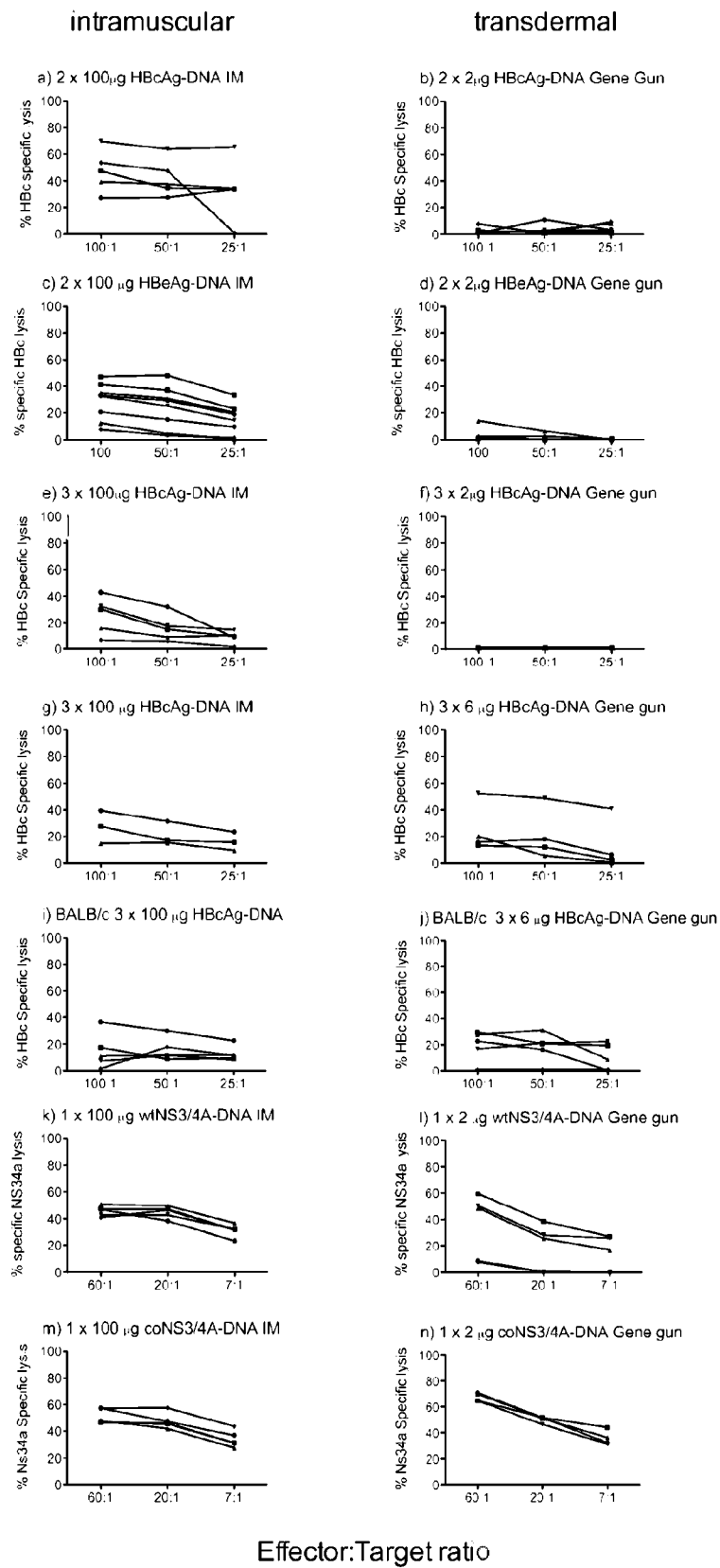


Figure 26

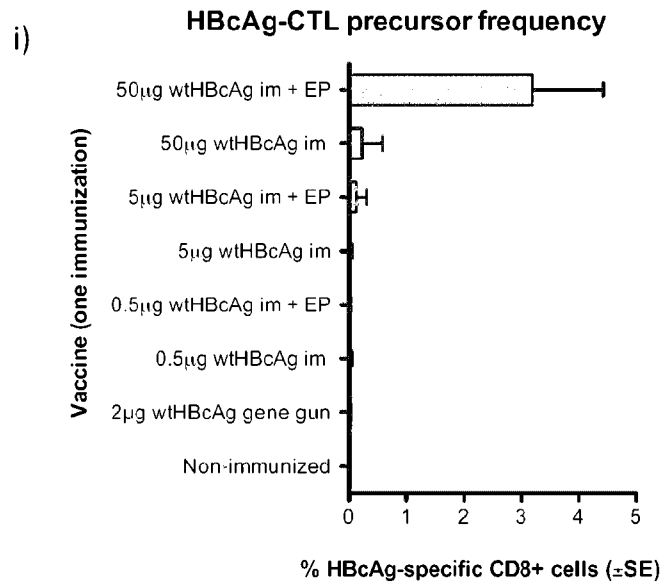
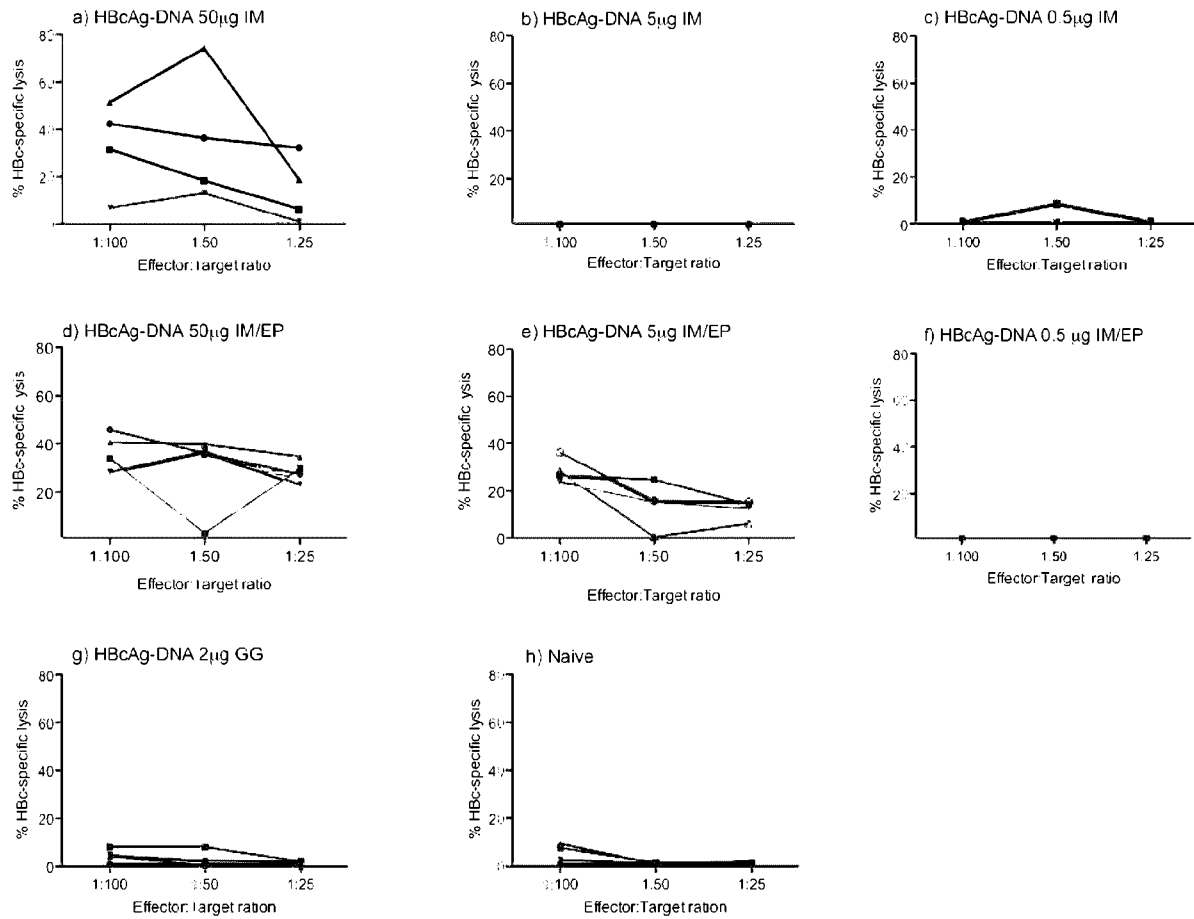
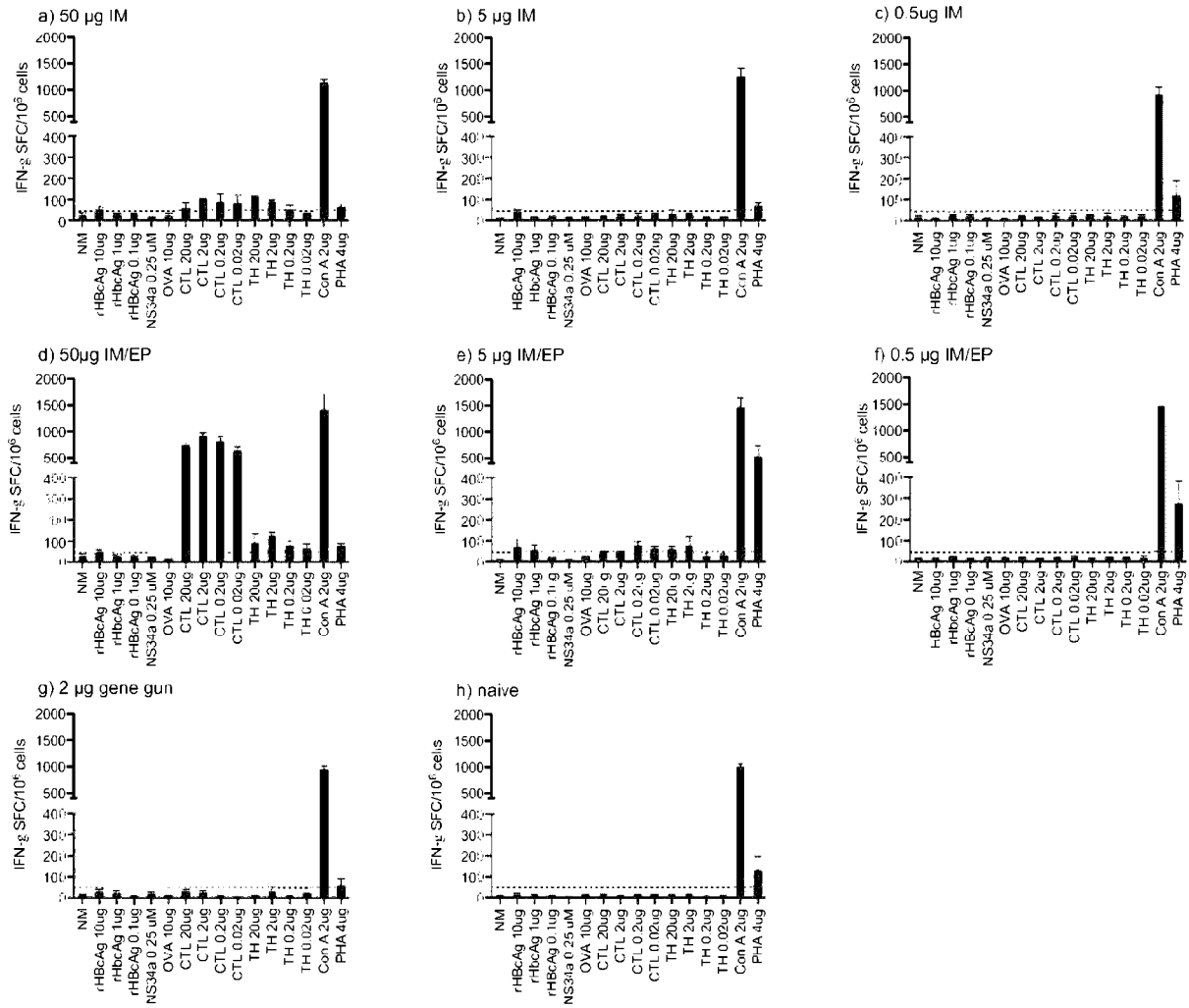


Figure 27



In vitro recall antigen

i) Clearance of HBcAg-expressing hepatocytes

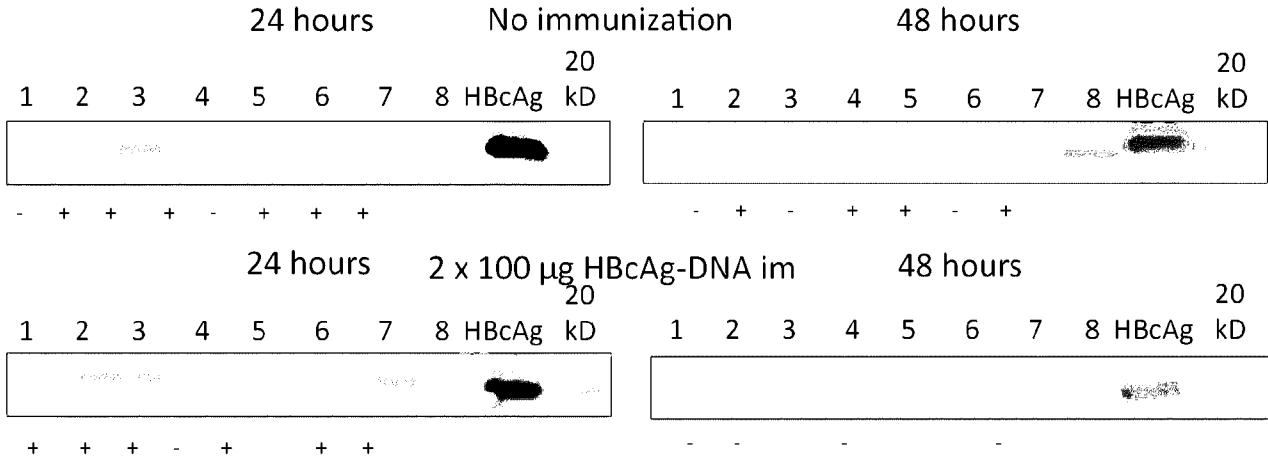


Figure 28

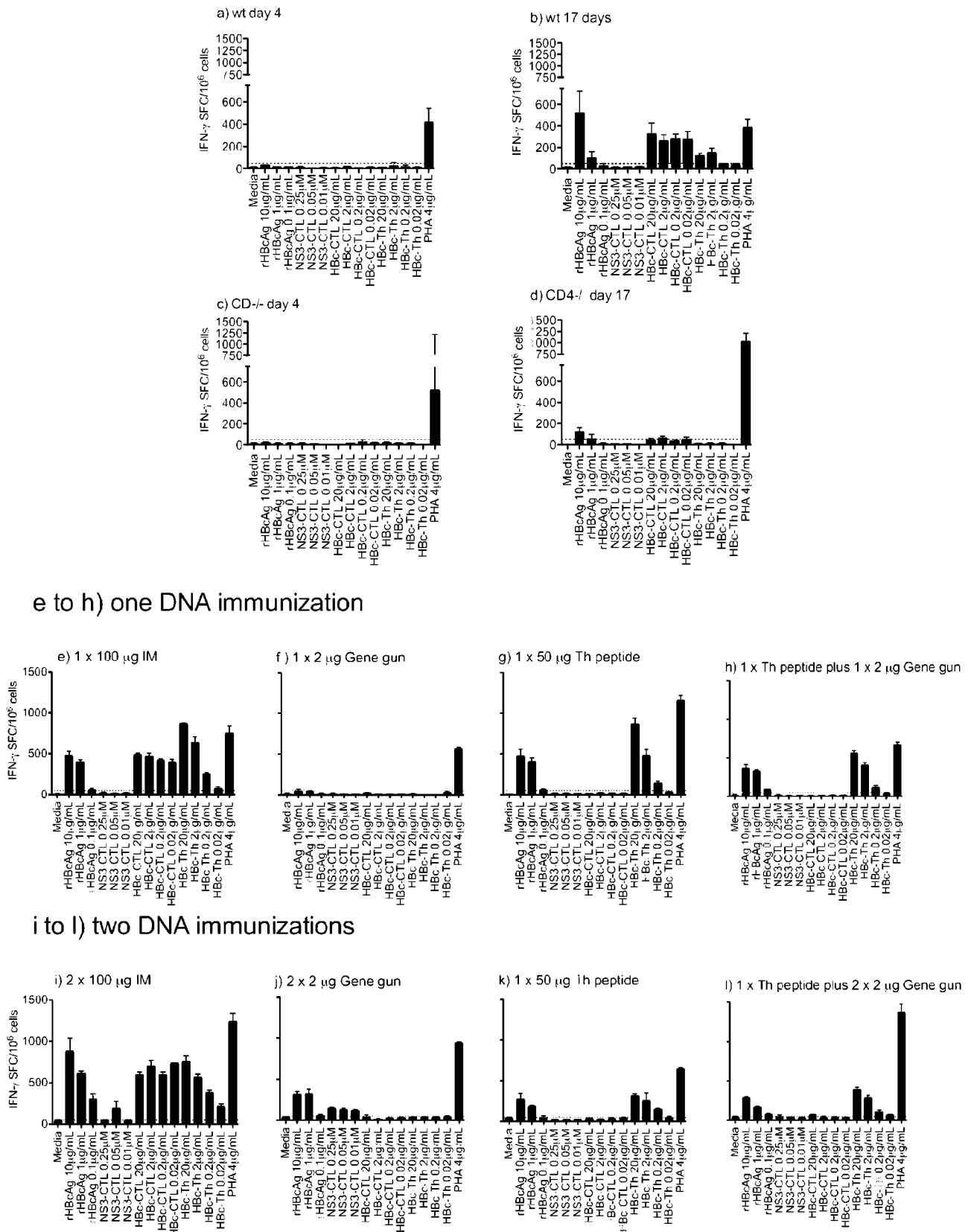


Figure 29

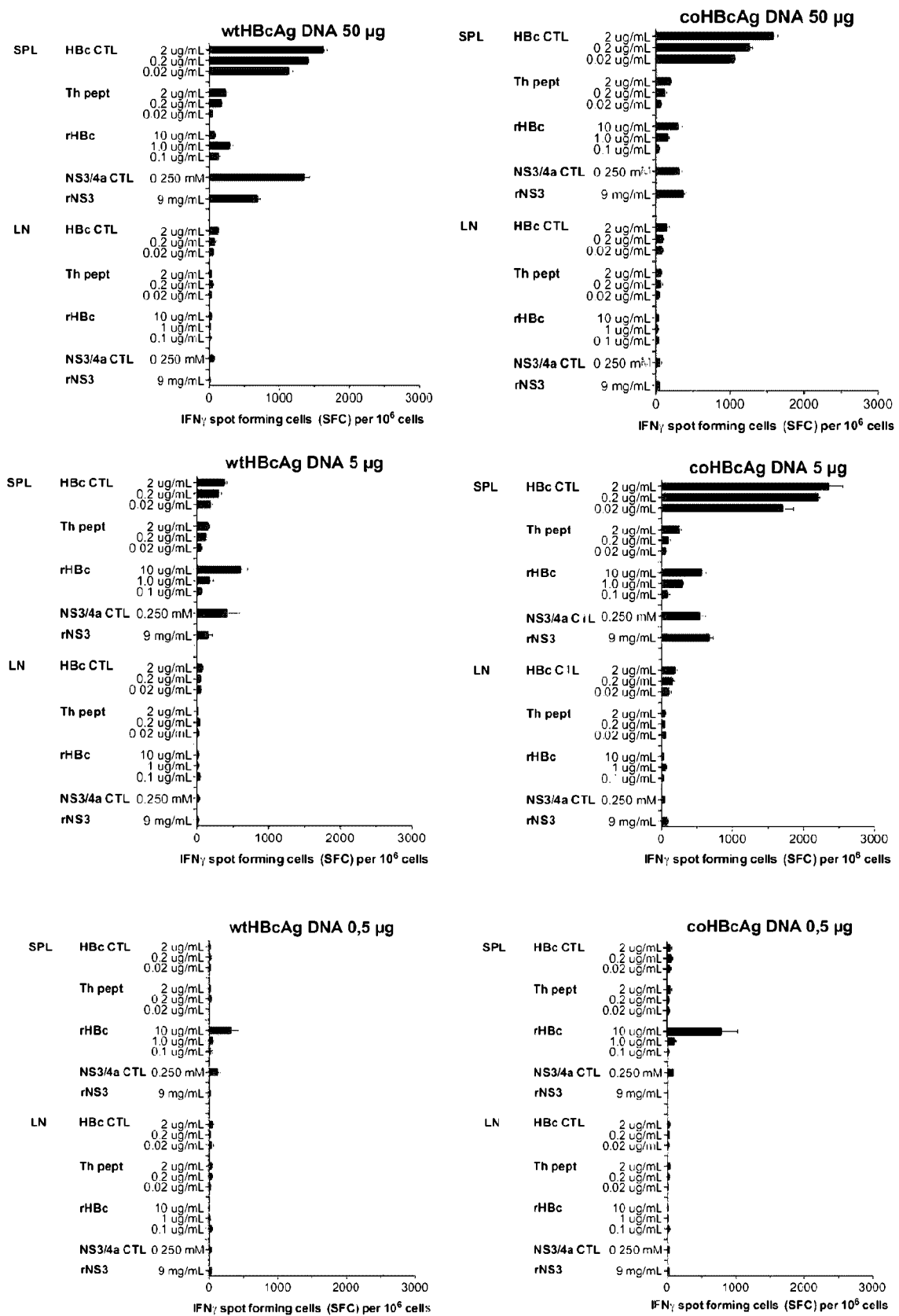


Figure 30

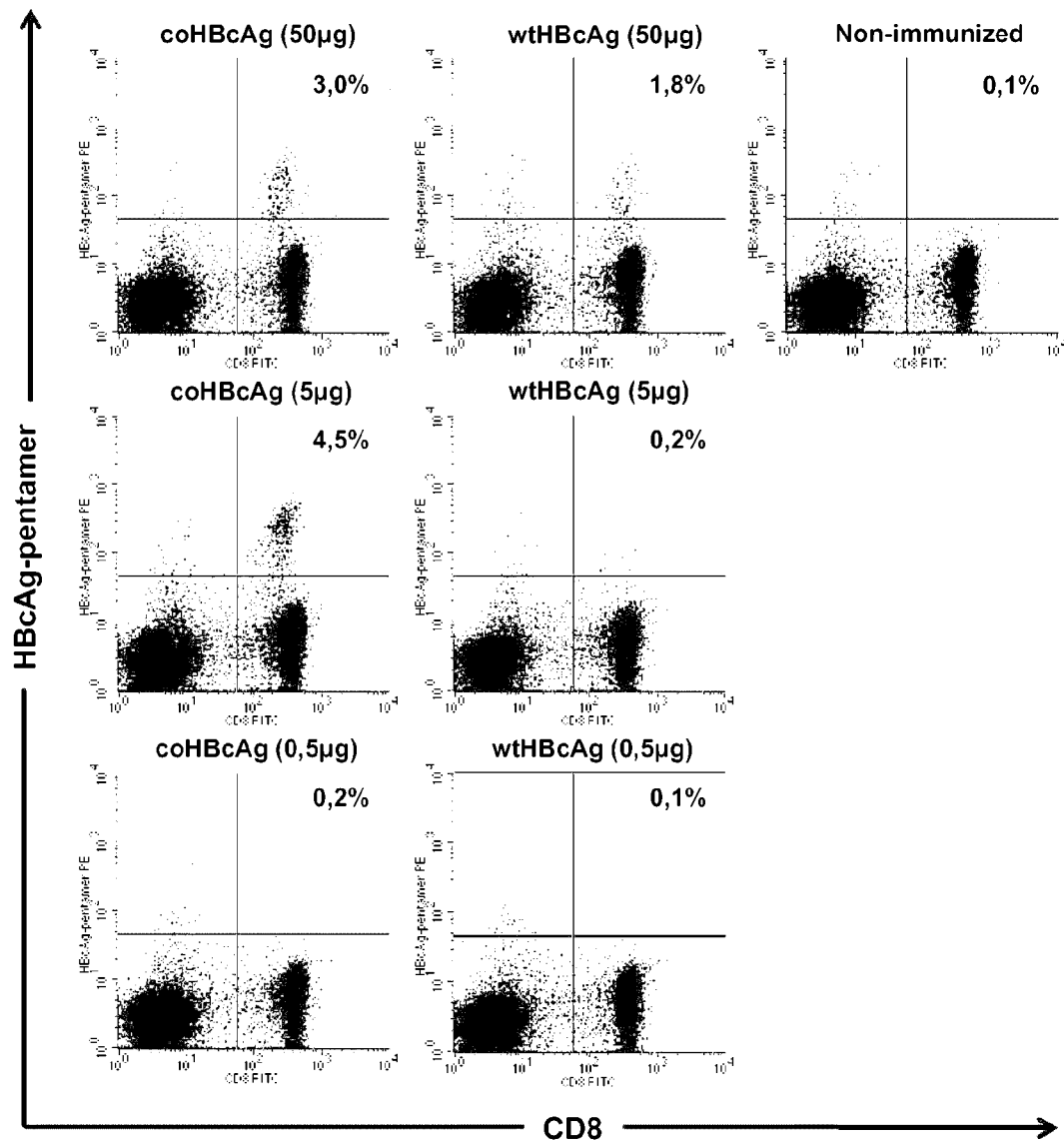


Figure 31

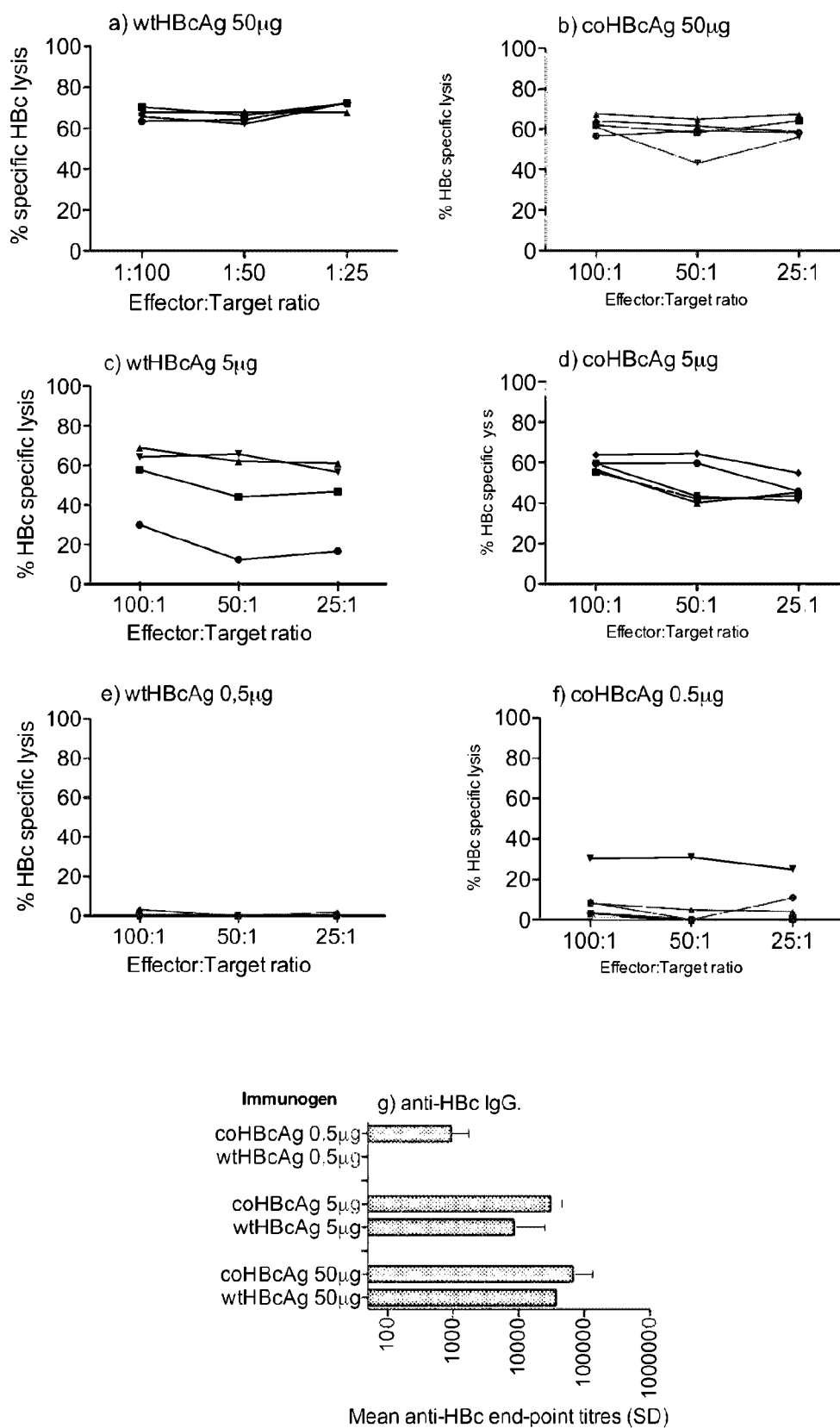


Figure 32