Abstract:
The invention provides a nucleic acid sequence encoding a chimeric adenovirus hexon protein, wherein the chimeric hexon protein comprises a first portion and a second portion. The first portion comprises at least 10 contiguous amino acid residues from a first adenovirus serotype (e.g., serotype 5 adenovirus hexon protein), optionally with one amino acid substitution. The second portion comprises (a) at least one hypervariable region (HVR) of a hexon protein of an adenovirus of a second adenovirus serotype, or (b) at least one synthetic hypervariable region (HVR) that is not present in the hexon protein of the wild-type adenovirus of the first adenovirus serotype.
ADENOVIRAL VECTORS WITH MODIFIED HEXON REGIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application claims the benefit of U.S. Provisional Patent Application No. 61/424,459, filed December 17, 2010, which is incorporated by reference.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 57,344 byte ASCII (text) file named "709216SequenceListing.txt," created on December 19, 2011.

BACKGROUND OF THE INVENTION

Recombinant adenoviral vectors have been used to transfer one or more recombinant genes to diseased cells or tissues in need of treatment. Adenoviral vectors are preferred over other vectors commonly employed for gene therapy (e.g., retroviral vectors) since adenoviral vectors can be produced in high titers (i.e., up to $10^{13}$ viral particles/ml), and efficiently transfer genes to nonreplicating, as well as replicating, cells (see, e.g., Crystal, Science, 270: 404-410 (1995)). Adenoviral vectors also are preferred because of their normal tropism for the respiratory epithelium and for other reasons (see, e.g., Straus, In Adenoviruses, Plenan Press, New York, N.Y., 451-496 (1984)); Horwitz et al., In Virology, 2nd Ed., Fields et al., eds., Raven Press, New York, N.Y., 1679-1721 (1990); Berkner, BioTechniques, 6: 616-629 (1988); Chanock et al., JAMA, 195: 151 (1966); Haj-Ahmad et al., J. Virol, 57: 267 (1986); and Ballay et al., EMBO, 4: 3861 (1985)).

There are 49 human adenoviral serotypes, categorized into 6 subgenera (A through F) based on nucleic acid comparisons, fiber protein characteristics, and biological properties (Crawford-Miksza et al., J. Virol., 70: 1836-1844 (1996)). The group C viruses (e.g., serotypes 2 and 5, or Ad2 and Ad5, respectively) are well characterized, and currently are employed for gene transfer studies, including human gene therapy trials (see, e.g., Rosenfeld et al, Science, 252: 431-434 (1991); Rosenfeld et al., Cell, 68: 143-155 (1992); Zabner, Cell, 75: 207-216 (1993); Crystal et al., Nat. Gen., 8: 42-51 (1994); Yei et al., Gene
Therapy, 1: 192-200 (1994); Chen et al, Proc. Natl Acad. Sci., 91: 3054-3057 (1994); Yang et al., Nat. Gen., 7: 362-369 (1994); Zabner et al, Nat. Gen., 6: 75-83 (1994)). Other groups and serotypes include, but are not limited to, group A (e.g., serotypes 12 and 31), group B (e.g., serotypes 3, 7, and 34), group D (e.g., serotypes 43 and 48), group E (e.g., serotype 4) and group F (e.g., serotypes 40 and 41) (Horwitz et al., supra).

One problem encountered with the use of adenoviral vectors for gene transfer in vivo is the generation of antibodies to antigenic epitopes on adenoviral capsid proteins. If sufficient in titer, the antibodies can limit the ability of the vector to be used more than once as an effective gene transfer vehicle. For instance, animal studies demonstrate that intravenous or local administration (e.g., to the lung, heart, or peritoneum) of an adenoviral type 2 or 5 gene transfer vector can result in the production of antibodies directed against the vector which prevent expression from the same serotype vector administered 1 to 2 weeks later (see, e.g., Yei et al., supra; Zabner (1994), supra; Setoguchi et al., Am. J. Respir. Cell. Mol Biol, 10: 369-377 (1994); Kass-Eisler et al., Gene Therapy, 1: 395-402 (1994); Kass-Eisler et al., Gene Therapy 3: 154-162 (1996)). This is a drawback in adenoviral-mediated gene therapy, since many uses of an adenoviral vector (e.g., for prolonged gene therapy) require repeat administration. The mechanism by which antibodies directed against an adenovirus are able to prevent or reduce expression of an adenoviral-encoded gene is unclear. This phenomenon is loosely referred to as "neutralization," and the responsible antibodies are termed "neutralizing antibodies."

Hexon proteins are the largest and most abundant proteins in the adenovirus capsid, making them a primary target for modification in order to reduce neutralization of adenoviral vectors (see, e.g., Gall et al., J. Virol, 72: 10260-264 (1998), and Rux et al, J. Virol, 77(17): 9553-9566 (2003)). However, many of the hexon modifications made to date have not been successful in sufficiently reducing the neutralizing antibody response. These failures are due, at least in part, to the disruption of structural interactions between the loops of the hexon protein, which interferes with the stability of the hexon structure itself, and likely impedes the ability of the hexon region to interact with other capsid proteins. In addition, many of the hexon modification made to date have adversely affected adenovirus growth.

Thus, there remains a need for modified hexon proteins which exhibit a decreased ability or inability to be recognized by antibodies (i.e., neutralizing antibodies) in vivo, and which do not adversely affect adenovirus growth. The invention provides nucleic acid
sequences encoding such hexon proteins, as well as adenoviral vectors comprising such hexon proteins.

BRIEF SUMMARY OF THE INVENTION

The invention provides a nucleic acid sequence encoding a chimeric adenovirus hexon protein. The chimeric hexon protein comprises (a) a first portion comprising at least 10 contiguous amino acid residues of hexon protein of a wild-type serotype 5 adenovirus, optionally with one amino acid substitution, and (b) a second portion comprising at least one hypervariable region (HVR) of a hexon protein of an adenovirus of a second adenovirus serotype, wherein the second adenovirus serotype is not serotype 5.

The invention also provides a nucleic acid sequence encoding a chimeric adenovirus hexon protein. The chimeric hexon protein comprises (a) a first portion comprising at least 10 contiguous amino acid residues of a hexon protein of a wild-type adenovirus of a first adenovirus serotype, optionally with one amino acid substitution, and (b) a second portion comprising at least one synthetic hypervariable region (HVR) that is not present in the hexon protein of any wild-type adenovirus.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Figure 1 is a schematic representation of serotype 5 adenoviral vectors carrying HVR substitutions in the hexon DEI and FGI hypervariable loops prepared as described in Example 1.

Figure 2 is a graph depicting the results of a focus forming unit assay conducted to measure adenoviral vector growth as described in Example 1.

Figure 3 is a graph depicting the results of a focus forming unit assay conducted to measure adenoviral vector growth as described in Example 2.

Figures 4A and 4B are graphs depicting the results of focus forming unit assays conducted to measure vector growth as described in Example 3. Figure 4A corresponds to 293 cells that were incubated at 32 °C, and Figure 4B corresponds to 293 cells that were incubated at 37 °C.

Figures 5A and 5B are graphs depicting the results of a focus forming unit assay conducted to measure vector growth as described in Example 4. Figure 5A corresponds to 293 cells that were incubated at 32 °C, and Figure 5B corresponds to 293 cells that were incubated at 37 °C.
Figure 6 is a graph which depicts experimental data illustrating the CD8+ T cell responses induced in mice by administration of hexon-modified adenoviral vectors with and without the T333M mutation in mice having pre-existing Ad5-specific neutralizing antibodies. Adnull351 1.1 ID is an El/E3/E4-deleted Ad5 vector that lacks a transgene. Adt.PyCSP1.1O is an El/E3-deleted Ad5 vector encoding PyCSP that is not hexon-modified. Adt.(PyCSP)H(5-43m.43) is an El/E3-deleted Ad5 vector which encodes PyCSP and contains a chimeric hexon protein in which all of the HVRs are replaced with HVRs from a serotype 43 adenovirus. Adt.(PyCSP)H(5-43m.43)sp is identical to Adt.(PyCSP)H(5-43m.43), except that it also contains the T333M point mutation. Adt.(PyCSP)H(5-48.48) is an El/E3-deleted Ad5 vector which encodes PyCSP and contains a chimeric hexon protein in which all of the HVRs are replaced with HVRs from a serotype 48 adenovirus. Adt.(PyCSP)H(5-48.48)sp is identical to Adt.(PyCSP)H(5-48.48), except that it also contains the T333M point mutation. Adt.PyCSP.1 ID is an El/E3/E4-deleted Ad5 vector encoding PyCSP that is not hexon-modified. Adt.(PyCSP)H(5-43m.43).1 ID is an El/E3/E4-deleted Ad5 vector which encodes PyCSP and contains a chimeric hexon protein in which all of the HVRs are replaced with HVRs from a serotype 43 adenovirus. T-cell responses were assessed by intracellular cytokine staining (ICS) of individual mice splenocytes. Targets were MHC-matched A20.2J cells pulsed with synthetic peptides representing the immunodominant CD8+ T cell epitope (PyCSP280-288), a subdominant epitope (PyCSP57-70), or a defined CD8+ T cell epitope for hemagglutinin (HA 332-340).

Figure 7 is a graph which depicts experimental data illustrating the PfCSP-specific antibody responses induced in mice by administration of the adenoviral vectors described in Example 4. Mice in groups (grp) 1-5 lacked Ad5-specific neutralizing antibodies, while mice in groups 6-9 exhibited Ad5-specific neutralizing antibodies. Hexon-modified adenoviral vectors carrying the T333M mutation, with or without a chimeric fiber protein, induce PfCSP-specific antibody.

Figure 8 is a graph which depicts experimental data illustrating the CD8+ T cell responses induced in mice by administration of the adenoviral vectors described in Example 4. "H-M CSP" refers to the El/E4-deleted Ad5 vector containing hexon HVR regions from Ad43 and the T333M mutation (AdPfCSP6H43.1 ID), and "HF-M CSP" refers to the E1/E4-deleted Ad5 vector containing hexon HVR regions from Ad43 and the T333M mutation, as well as a chimeric fiber protein comprising an Ad5 tail and shaft region and a knob region from a serotype 25 simian adenovirus (AdPfCSP6H43spF25SK.1 ID).
Figure 9 is a graph which depicts experimental data illustrating norovirus-specific antibody titers induced by administration of the adenoviral vectors described in Example 5. The error bars represent 95% confidence intervals.

Figure 10 is a graph which depicts experimental data illustrating the CD8+ T cell responses induced in mice by administration of the adenoviral vectors encoding an Epstein Barr Virus (EBV) antigen described in Example 6.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides an isolated or purified nucleic acid sequence which encodes a chimeric adenovirus hexon protein. The chimeric adenovirus hexon protein comprises, or alternatively consists of, a first portion related to the wild-type hexon protein of an adenovirus of a first adenovirus serotype and either a second portion related to the wild-type hexon protein of an adenovirus of a second adenovirus serotype that differs from the first adenovirus serotype or a second portion that is a synthetic sequence that is not present in the wild-type hexon protein of the first adenovirus serotype. With the exception of the chimeric hexon protein, the genome of an adenoviral vector as described herein containing the chimeric hexon protein, is based on an adenovirus of the first adenovirus serotype unless otherwise indicated.

In particular, the invention provides a nucleic acid sequence encoding a chimeric adenovirus hexon protein, wherein the chimeric hexon protein comprises, or consists of, (a) a first portion comprising, or consisting of, at least 10 contiguous amino acid residues of hexon protein of a wild-type serotype 5 adenovirus, optionally with one amino acid substitution, and (b) a second portion comprising, or consisting of, at least one hypervariable region (HVR) of a hexon protein of an adenovirus of a second adenovirus serotype, wherein the second adenovirus serotype is not serotype 5. The invention also provides a nucleic acid sequence encoding a chimeric adenovirus hexon protein, wherein the chimeric hexon protein comprises, or consists of, (a) a first portion comprising, or consisting of, at least 10 contiguous amino acid residues of a hexon protein of a wild-type adenovirus of a first adenovirus serotype, optionally with one amino acid substitution, and (b) a second portion comprising, or consisting of, at least one synthetic hypervariable region (HVR) that is not present in the hexon protein of any wild-type adenovirus.

The chimeric hexon protein desirably is generated by replacing one or more portions of an amino acid sequence of a wild-type hexon protein of an adenovirus of a first
serotype (e.g., a serotype 5 adenovirus) with either (a) one or more portions of an amino acid sequence of a wild-type hexon protein of an adenovirus of a second, and different, serotype (e.g., an adenovirus of a serotype other than serotype 5) or (b) one or more synthetic amino acid sequences that are not present in the wild-type hexon protein of the adenovirus of the first serotype (e.g., a serotype 5 adenovirus). The aforementioned replacement in the wild-type hexon protein of the adenovirus of the first serotype desirably is effected by making the appropriate changes in the wild-type nucleic acid sequence encoding the hexon protein of the adenovirus of the first serotype, such that the modified nucleic acid sequence encodes the chimeric hexon protein described herein.

The terms "nucleic acid sequence," "nucleic acid," "nucleic acid molecule," and "polynucleotide" encompass a polymer of DNA or RNA, i.e., a polynucleotide, which can be single-stranded or double-stranded and which can contain non-natural or altered nucleotides. In this respect, these terms include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs and modified polynucleotides such as, though not limited to, methylated and/or capped polynucleotides.

By "isolated" is meant the removal of a nucleic acid from its natural environment. By "purified" is meant that a given nucleic acid, whether one that has been removed from nature (including genomic DNA and mRNA) or synthesized (including cDNA) and/or amplified under laboratory conditions, has been increased in purity, wherein "purity" is a relative term and does not mean absolute purity. It is to be understood, however, that nucleic acids and proteins may be formulated with diluents or adjuvants and nevertheless for practical purposes be isolated.

The hexon protein is "chimeric" in that it comprises a sequence of amino acid residues that is not typically found in a hexon protein as isolated from, or identified in, a wild-type adenovirus, which comprises the so-called native hexon protein or "wild-type hexon protein." The chimeric hexon protein thus comprises (or has) a "nonnative amino acid sequence." By "nonnative amino acid sequence" is meant any amino acid sequence (i.e., either component residues or order thereof) that is not found in the native hexon protein of a given serotype of adenovirus, and which preferably is introduced into the hexon protein at the level of gene expression (i.e., by production of a nucleic acid sequence that encodes the nonnative amino acid sequence).

By "portion" is meant an amino acid sequence that comprises at least three amino acids (e.g., about 3 to about 800 amino acids). Preferably, a "portion" comprises 10 or more
(e.g., 10 or more, 15 or more, 20 or more, 25 or more, 30 or more, 40 or more, 50 or more, or
100 or more) amino acid residues, but less than the entire wild-type hexon protein (e.g., 800
or less, 700 or less, 600 or less, 500 or less, 400 or less, 300 or less, 200 or less, or 100 or less
amino acid residues). For example, a portion can be about 10 to about 500 amino acids (e.g.,
about 10, 100, 300, or 500 amino acids), about 10 to about 300 amino acids (e.g., about 20,
50, or 200 amino acids), or about 10 to about 100 amino acids (e.g., about 15, 40, 60, 70, or
90 amino acids), or a range defined by any two of the foregoing values. More preferably, a
"portion" comprises no more than about 300 amino acids (e.g., about 10 to about 250 amino
acids, about 10 to about 200 amino acids, or about 50 to about 100 amino acids, or a range
defined by any two of the foregoing values).

[0027] The first portion of the chimeric hexon protein comprises at least 10 contiguous
amino acid residues of a hexon protein of a wild-type adenovirus of a first adenovirus
serotype, optionally with one amino acid substitution. The first adenovirus serotype from
which the first portion of the chimeric hexon protein is derived or obtained from can be any
adenovirus serotype described herein, but preferably the first adenovirus serotype is serotype
5. The first adenovirus serotype can be a human or non-human adenovirus serotype. In a
preferred embodiment, the first adenovirus serotype is human serotype 5.

[0028] When the first portion of the chimeric hexon protein comprises at least 10
contiguous amino acid residues of the hexon protein of the wild-type serotype 5 adenovirus
with one amino acid substitution, the first portion include a point mutation, i.e., a single
amino acid residue replacement, as compared to the corresponding portion of the hexon
protein of the wild-type serotype 5 adenovirus. The point mutation can be effected in any
suitable manner. Desirably, the nucleic acid sequence which encodes the first portion of the
chimeric hexon protein comprises a mutation, e.g., a replacement of one or more nucleotides,
that results in a single amino acid substitution in the protein encoded thereby. The nucleic
acid sequence can be mutated by any suitable method known in the art, such as, for example,
by insertion, deletion, and/or substitution. For example, mutations may be introduced into a
nucleic acid sequence randomly or in a site-specific manner. Random mutations may be
generated, for example, by error-prone PCR of a template sequence. A preferred means for
introducing random mutations in is the Genemorph II Random Mutagenesis Kit (Stratagene,
La Jolla, CA). Site-specific mutations can be introduced, for example, by ligating into an
expression vector a synthesized oligonucleotide comprising the modified site. Alternately,
oligonucleotide-directed site-specific mutagenesis procedures can be used, such as those
disclosed in Walder et al., Gene, 42: 133 (1986); Bauer et al., Gene, 37: 73 (1985); Craik, Biotechniques, 12-19 (January 1995); and U.S. Patents 4,518,584 and 4,737,462. A preferred means for introducing site-specific mutations is the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

[0029] The amino acid substitution can be of any amino acid residue with any other amino acid residue. As such, the amino acid substitution can be a conservative amino acid substitution or mutation, a semi-conservative substitution or mutation, or a non-conservative substitution or mutation. The phrase "conservative amino acid substitution" or "conservative mutation" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag, New York (1979)). According to such analyses, groups of amino acids may be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. and R. H. Schirmer, supra). Examples of conservative mutations include amino acid substitutions of amino acids within the sub-groups described below, for example, lysine for arginine and vice versa such that a positive charge may be maintained; glutamic acid for aspartic acid and vice versa such that a negative charge may be maintained; serine for threonine such that a free -OH can be maintained; and glutamine for asparagine such that a free -NH$_2$ can be maintained. "Semi-conservative substitutions" or "semi-conservative mutations" include amino acid substitutions of amino acids with the same groups listed below, but that do not share the same sub-group. For example, the mutation of aspartic acid for asparagine, or asparagine for lysine, each involves amino acids within the same group, but different sub-groups. "Non-conservative substitutions" or "non-conservative mutations" involve amino acid substitutions between different groups, for example lysine for tryptophan, or phenylalanine for serine.

[0030] Amino acids are broadly grouped as "aromatic" or "aliphatic." An aromatic amino acid includes an aromatic ring. Examples of "aromatic" amino acids include histidine (H or His), phenylalanine (F or Phe), tyrosine (Y or Tyr), and tryptophan (W or Trp). Non-aromatic amino acids are broadly grouped as "aliphatic." Examples of "aliphatic" amino acids include glycine (G or Gly), alanine (A or Ala), valine (V or Val), leucine (L or Leu), isoleucine (I or Ile), methionine (M or Met), serine (S or Ser), threonine (T or Thr), cysteine
(C or Cys), proline (P or Pro), glutamic acid (E or Glu), aspartic acid (A or Asp), asparagine (N or Asn), glutamine (Q or Gin), lysine (K or Lys), and arginine (R or Arg).

Aromatic amino acids may be sub-divided into two sub-groups: the "nitrogen ring sub-group" consisting of histidine and tryptophan, and the "phenyl sub-group" consisting of phenylalanine and tyrosine.

Aliphatic amino acids may be sub-divided into four sub-groups. The "large aliphatic non-polar sub-group" consists of valine, leucine and isoleucine, the "aliphatic slightly-polar sub-group" consists of methionine, serine, threonine, and cysteine, the "aliphatic polar/charged sub-group" consists of glutamic acid, aspartic acid, asparagine, glutamine, lysine, and arginine, and the "small-residue sub-group" consists of glycine and alanine. The group of charged/polar amino acids may be sub-divided into three sub-groups: the "positively-charged sub-group," consisting of lysine and arginine, the "negatively-charged sub-group," consisting of glutamic acid and aspartic acid, and the "polar sub-group" consisting of asparagine and glutamine.

The point mutation described herein can result in an amino acid substitution at any suitable residue. Preferably, the amino acid substitution is at residue 342 of the hexon protein of the wild-type serotype 5 adenovirus. The numbering of amino acid positions in the chimeric hexon protein used herein is based on the hexon amino acid sequence including the initial methionine residue. The point mutation described herein also can result in a conservative, semi-conservative, or non-conservative amino acid substitution as described herein. Preferably, the amino acid substitution is a threonine (T) to methionine (M) substitution.

The second portion of the chimeric hexon protein comprises at least one HVR that is not present in the hexon protein of the wild-type adenovirus of the first adenovirus serotype.

In a first embodiment of the second portion, the second portion of the chimeric hexon protein comprises at least one HVR of a hexon protein of an adenovirus of a second adenovirus serotype, wherein the second adenovirus serotype is not the same as the first adenovirus serotype (i.e., wherein the first and second serotypes are different). Thus, when the first adenovirus serotype is serotype 5, the second adenovirus serotype is not serotype 5. The second portion of the chimeric hexon protein desirably comprises at least one HVR that occurs naturally in a hexon protein of a wild-type adenovirus (i.e., a wild-type hexon protein)
of a second serotype that differs from the first serotype (e.g., a second serotype that is not serotype 5).

The one or more HVRs of the wild-type hexon protein of the second adenovirus serotype desirably replace one or more HVRs of the wild-type hexon protein of the first adenovirus serotype in providing the chimeric hexon protein. The HVRs of the hexon protein are located in the loops of the hexon protein (DEI and FG1), which are found at the top of the hexon molecule (see, e.g., Rux et al., *J. Virol*, 77(17): 9553-9566 (2003)). The hypervariable regions vary in length and sequence between adenoviral serotypes (Crawford-Miksza et al., *J. Virol*, 70: 1836-1844 (1996)). The HVR regions include the HVR1 region, the HVR2 region, the HVR3 region, the HVR4 region, the HVR5 region, the HVR6 region, the HVR7 region, the HVR8 region, and the HVR9 region.

The amino acid sequence of a wild-type serotype 5 hexon protein comprises SEQ ID NO: 1. Preferably, amino acid residues within the FG1, FG2, or DEI loops of a hexon protein of a serotype 5 adenovirus are deleted and replaced with corresponding amino acid residues from a hexon protein of a second, and different, adenovirus serotype (i.e., corresponding amino acid residues from a hexon protein of a wild-type adenovirus of a serotype other than serotype 5). An entire loop region can be removed from a hexon protein of a wild-type serotype 5 adenovirus and replaced with the corresponding loop region of a hexon protein of a wild-type adenovirus of a serotype other than serotype 5. Alternatively, one or more portions of a loop region can be removed from the hexon protein of a wild-type serotype 5 adenovirus and replaced with one or more corresponding portions of a loop region of a hexon protein of a wild-type adenovirus of a serotype other than serotype 5. Similarly, one or more hexon loops, or portions thereof, of a hexon protein of a wild-type serotype 5 adenovirus can be removed and replaced with the corresponding amino acid sequences of a hexon protein of a wild-type adenovirus of a serotype other than serotype 5. Preferably, the second adenovirus serotype is serotype 43, 48, or 34. Thus, in one embodiment, one or more hexon loops, or portions thereof, of a hexon protein of a wild-type serotype 5 adenovirus are removed and replaced with corresponding amino acid sequences of a hexon protein of a wild-type adenovirus of serotype 43, 48, or 34. For example, the chimeric hexon protein can have the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. In a preferred embodiment, all nine HVRs of a hexon protein of a wild-type serotype 5 adenovirus are removed and replaced with the corresponding amino acid sequences of a hexon protein of a wild-type adenovirus of serotype 43. The structure of
serotype 5 hexon proteins and methods of modifying hexon proteins are disclosed in, for example, Rux et al, J. Virol, 77(17): 9553-9566 (2003), and U.S. Patent 6,127,525.

[0038] The second portion of the chimeric hexon protein can comprise any HVR of the wild-type adenovirus of the second serotype, as well as any number of HVRs of the wild-type adenovirus of the second adenovirus serotype. For example, the second portion of the chimeric hexon protein can comprise one or more of HVR1, HVR2, HVR3, HVR4, HVR5, HVR6, HVR7, HVR8, and HVR9 of a hexon protein of a wild-type adenovirus of the second serotype. The second portion of the chimeric hexon protein preferably comprises at least one HVR of the DEI loop and/or FG1 loop of a wild-type hexon protein of the second adenovirus serotype (e.g., 1, 2, 3, 4, 5, 6, 7, 8, or 9 HVRs). More preferably, the second portion of the chimeric hexon protein comprises three or more HVRs of the DEI loop and/or FG1 loop of a wild-type hexon protein of the second adenovirus serotype (e.g., 3, 4, 5, 6, 7, 8, or 9 HVRs).

In a particularly preferred embodiment, the second portion of the chimeric hexon protein comprises six HVRs of the DEI loop of a wild-type hexon protein of the second adenovirus serotype and/or three HVRs of the FG1 loop of a wild-type hexon protein of the second adenovirus serotype. Thus, for example, a region of a wild-type hexon protein of adenovirus serotype 5 (Ad5) comprising HVR1-HVR6 can be deleted and replaced with a region comprising HVR1-HVR6 of a hexon protein of a wild-type adenovirus of serotype 43 (Ad43). In another example, a region of a wild-type hexon protein of adenovirus serotype 5 (Ad5) comprising HVR1-HVR9 can be deleted and replaced with a region of a wild-type hexon protein of adenovirus serotype 48 (Ad48) comprising HVR1-HVR9.

[0039] As is apparent from the foregoing description, the second portion of the chimeric hexon protein can comprise any portion of the hexon protein, in addition to the at least one HVR, of the wild-type adenovirus of the second serotype. For example, the second portion of the chimeric hexon protein can comprise the entirety of the DEI and/or FG1 loops of a hexon protein of a wild-type adenovirus of a second serotype.

[0040] The second portion of the chimeric hexon protein optionally further comprises at least one HVR that is synthetically-generated (i.e., a "synthetic HVR"). By "synthetic HVR" is meant that the HVR does not occur naturally in a wild-type hexon protein of an adenovirus of any serotype and preferably is generated using routine biochemical techniques. In this respect, a synthetic HVR can be a native adenovirus HVR that has been modified in any suitable manner, e.g., by deletion, insertion, or substitution of one or more amino acid residues, using routine molecular biology methods known in the art. Preferably, however, a
synthetic HVR is not derived from an adenovirus. In this respect, the HVR can be a synthetically-generated random amino acid sequence that comprises about 1 to about 40 amino acid residues (e.g., about 1, 5, 10, 15, 20, 25, 30, 35, or 40 amino acid residues, or a range defined by any two of the foregoing values). A preferred random amino acid sequence is one that has been selected from among several other random amino acid sequences because the random amino acid sequence does not impede, and preferably enhances, growth of an adenoviral vector and/or because the random amino acid sequence reduces host immune responses directed against the chimeric hexon protein. Thus, a random amino acid sequence also can be referred to as a "selected random amino acid sequence."

[0041] In a preferred embodiment, the second portion of the chimeric hexon protein comprises two or more HVRs, with at least one HVR being a synthetic HVR, especially wherein a random amino acid sequence desirably is utilized as at least one synthetic HVR. In other words, in such a preferred embodiment, the second portion of the chimeric hexon protein comprises one or more wild-type HVRs of the second adenovirus serotype and one or more synthetic HVRs, wherein at least one synthetic HVR desirably is a synthetically-derived random amino acid sequence as described herein.

[0042] The synthetic HVR can be an epitope of a pathogen. Therefore, the second portion of the chimeric hexon protein can comprise an epitope of a pathogen in place of at least one wild-type HVR of the second serotype. It will be appreciated that incorporating an epitope of a pathogen into the adenovirus capsid may enhance the immune response elicited by an adenoviral vector comprising the hexon protein when administered to a mammal as a vaccine. By "epitope" is meant a sequence on an antigen that is recognized by an antibody or an antigen receptor. Epitopes also are referred to in the art as "antigenic determinants." An "antigen" is a molecule that induces an immune response in a mammal. An "immune response" can entail, for example, antibody production and/or the activation of immune effector cells (e.g., T cells). An antigen in the context of the invention can comprise any subunit, fragment, or epitope of any proteinaceous molecule, including a protein or peptide of viral, bacterial, parasitic, fungal, protozoan, prion, cellular, or extracellular origin, which ideally provokes an immune response in mammal, preferably leading to protective immunity.

[0043] A "pathogen" is an infectious agent that causes disease to its host. Suitable pathogens include, for example, viruses, bacteria, parasites, fungi, protozoa, or prions. In one embodiment, the pathogen is a virus. The epitope can be isolated from any virus including, but not limited to, a virus from any of the following viral families: Arenaviridae, Arterivirus,
Astroviridae, Baculoviridae, Badnavirus, Barnaviridae, Birnaviridae, Bromoviridae, Bunyaviridae, Caliciviridae (e.g., Norovirus (also known as "Norwalk-like virus")), Capillovirus, Carlaviridae, Caulimovirus, Circoviridae, Closterovirus, Comoviridae, Coronaviridae (e.g., Coronavirus, such as severe acute respiratory syndrome (SARS) virus), Corticoviridae, Cystoviridae, Deltavirus, Dianthovirus, Enamovirus, Filoviridae (e.g., Marburg virus and Ebola virus (e.g., Zaire, Reston, Ivory Coast, or Sudan strain)), Flaviviridae, (e.g., Hepatitis C virus, Dengue virus 1, Dengue virus 2, Dengue virus 3, and Dengue virus 4), Hepadnaviridae (e.g., Hepatitis B virus or Hepatitis C virus), Herpesviridae (e.g., Human herpesvirus (HSV) 1, 2, 3, 4, 5, and 6, Cytomegalovirus, and Epstein-Barr Virus (EBV)), Hypoviridae, Iridoviridae, Leviridae, Lipothrixviridae, Microviridae, Orthomyxoviridae (e.g., Influenzavirus A and B), Papaviridae, Papillomaviridae (e.g., human papillomavirus (HPV)), Paramyxoviridae (e.g., measles, mumps, and human respiratory syncytial virus (RSV)), Parvoviridae, Picornaviridae (e.g., poliovirus, rhinovirus, hepatovirus, and aphthovirus), Poxviridae (e.g., vaccinia virus), Reoviridae (e.g., rotavirus), Retroviridae (e.g., lentivirus, such as human immunodeficiency virus (HIV) 1 and HIV 2), Rhabdoviridae, and Totiviridae. Particularly preferred retroviridae (retrovirus) antigens include, for example, HIV antigens, such as all or part of the gag, env, or pol proteins, or fusion proteins comprising all or part of the gag, env, or pol proteins. Any clade of HIV is appropriate for antigen selection, including clades A, B, C, MN, and the like. The viral antigen also can be an RSV antigen (e.g., all or part of an RSV F or G protein). Suitable viral antigens also include all or part of Dengue protein M, Dengue protein E, Dengue D1NS1, Dengue D1NS2, and Dengue D1NS3. The antigenic peptides specifically recited herein are merely exemplary as any viral protein can be used in the context of the invention.

Alternatively or in addition, the epitope can be isolated from a bacterial antigen. The antigen can originate from any bacterium including, but not limited to, Actinomyces, Anabaena, Bacillus, Bacteroides, Bdellovibrio, Caulobacter, Chlamydia (e.g., Chlamydia trachomatis), Chlorobium, Chromatium, Clostridium, Cytophaga, Deinococcus, Escherichia, Halobacterium, Heliobacter, Hyphomicrobium, Methanobacterium, Micrococcus, Myobacterium (e.g., Mycobacterium tuberculosis), Mycoplasma, Myxococcus, Neisseria, Nitrobacter, Oscillatoria, Prochloron, Proteus, Pseudomonas, Phodopirillum, Rickettsia, Salmonella, Shigella, Spirillum, Spirochaeta, Staphylococcus, Streptococcus, Streptomyces, Sulfolobus, Thermoplasma, Thiobacillus, and Treponema.
In another embodiment, the epitope can be isolated from a parasite such as, but not limited to, a parasite of the phylum *Sporozoa* (also referred to as phylum *Apicomplexa*), *Ciliophora, Rhizopoda,* or *Zoomastigophora.* Preferably, the epitope is from an antigen of a parasite of the phylum *Sporozoa* and genus *Plasmodium.* The antigen can be from any suitable *Plasmodium* species, but preferably is from a *Plasmodium* species that infects humans and causes malaria. Human-infecting *Plasmodium* species include *P. malariae, P. ovale, P. vivax,* and *P. falciparum.*

Desirably, the second portion of the chimeric hexon protein comprises an epitope of a pathogen in place of one wild-type HVR of the second adenovirus serotype. In other words, the second portion of the chimeric hexon protein desirably comprises one or more wild-type HVRs of the second adenovirus serotype and one or more synthetic HVRs, wherein at least one synthetic HVR is an epitope of a pathogen as described herein. Preferably, the second portion of the chimeric hexon protein comprises at least one wild-type HVR of the second adenovirus serotype and at least two synthetic HVRs, wherein each of the at least two synthetic HVRs is an epitope. In this embodiment, the same epitope can be utilized as each synthetic HVR, or different epitopes from the same or different pathogens can be used as the synthetic HVRs. For example, the second portion of the chimeric hexon protein can comprise six HVRs of the DEI loop of a wild-type hexon protein of the second adenovirus serotype, except that HVR1, HVR5, or both HVR1 and HVR5 are replaced with an epitope wherein the chimeric hexon protein comprises an epitope of a pathogen in place of HVR1 and/or HVR5. Epitope amino acid sequences can be inserted into the second portion of the chimeric hexon protein by effecting the removal of amino acid residues within one or more of the hypervariable regions of the second portion and addition of the epitope amino acid sequence (i.e., replacement of the removed amino acid residues with the epitope amino acid sequence) using methods described herein.

In a second embodiment of the second portion, the second portion of the chimeric hexon protein comprises at least one synthetic HVR that is not present in the hexon protein of any wild-type adenovirus. Preferably, the second portion of the chimeric hexon protein comprises two or more synthetic HVRs (e.g., 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, or 9 synthetic HVRs, or a range defined by any two of the foregoing values) that are not present in the hexon protein of any wild-type adenovirus. More preferably, the second portion of the chimeric hexon protein comprises three synthetic HVRs that are not present in the hexon protein of any wild-type adenovirus. Most preferably, the second
portion of the chimeric hexon protein comprises nine synthetic HVRs that are not present in the hexon protein of any wild-type adenovirus. The synthetic HVR can be any suitable synthetic HVR described herein, such a random amino acid sequence or an epitope of a pathogen.

The one or more synthetic HVRs desirably replace one or more HVRs of the wild-type hexon protein of the first adenovirus serotype in providing the chimeric hexon protein. As described above, the HVRs of the hexon protein are located in the loops of the hexon protein (DEI and FG1), which are found at the top of the hexon molecule (see, e.g., Rux et al., J. Virol, 77(17): 9553-9566 (2003)). The hypervariable regions vary in length and sequence between adenoviral serotypes (Crawford-Miksza et al., J. Virol, 70: 1836-1844 (1996)). The HVR regions include the HVR1 region, the HVR2 region, the HVR3 region, the HVR4 region, the HVR5 region, the HVR6 region, the HVR7 region, the HVR8 region, and the HVR9 region. Thus, for example, a region of a wild-type hexon protein of adenovirus serotype 5 (Ad5) comprising HVR1-HVR6 can be deleted and replaced with synthetic HVRs as described herein. In another example, a region of a wild-type hexon protein of adenovirus serotype 5 (Ad5) comprising HVR1-HVR9 can be deleted and replaced synthetic HVRs as described herein.


The invention also provides a chimeric adenovirus hexon protein encoded by the isolated or purified nucleic acid sequence described herein. The chimeric adenovirus hexon protein has a decreased ability or an inability to be recognized by an antibody (e.g., a neutralizing antibody) directed against a corresponding wild-type hexon protein of an adenovirus. A "neutralizing antibody" is an antibody that is purified from, or is present in, serum. As used herein, an antibody can be a single antibody or a plurality of antibodies. An antibody is "neutralizing" if the antibody inhibits infectivity of (i.e., cell entry by), or gene
expression commanded by, an adenovirus comprising a wild-type hexon protein, or if the antibody exerts a substantial deleterious effect on infectivity of, or gene expression commanded by, an adenovirus comprising a wild-type hexon protein. In one embodiment, the removal of one or more epitopes for a neutralizing antibody present in a wild-type hexon protein of an adenovirus to generate a chimeric adenovirus hexon protein will result in a decreased ability or inability of the chimeric hexon protein to be recognized by the neutralizing antibody.

[0051] An ability or inability of a chimeric hexon protein to "be recognized by" (i.e., interact with) a neutralizing antibody directed against a wild-type hexon protein of an adenovirus can be assessed by a variety of means known to those skilled in the art. For example, a decreased ability or inability to interact with a neutralizing antibody directed against wild-type hexon protein can be demonstrated by means of a neutralization test (see, e.g., Crawford-Miksza et al., supra; Mastrangeli et al., Human Gene Therapy, 7: 79-87 (1996)), or as further described herein.

[0052] Generally, an "inability" of a chimeric adenovirus hexon protein to be recognized by a neutralizing antibody directed against a wild-type hexon protein of an adenovirus means that such an antibody does not interact with the chimeric hexon protein and/or exhibits no substantial deleterious effect on infectivity of, or gene expression commanded by, an adenovirus comprising the chimeric hexon protein.

[0053] A "decreased ability" to be recognized by a neutralizing antibody directed against a wild-type hexon protein of an adenovirus refers to any decrease in the ability of the chimeric adenovirus hexon protein to be recognized by an antibody directed against a wild-type hexon protein of an adenovirus of the first adenovirus serotype, as described herein. When such ability/inability is assessed by means of a neutralization test in particular, preferably a "decreased ability" to be recognized by a neutralizing antibody directed against a wild-type hexon protein of an adenovirus of the first adenovirus serotype reflects about a 10% to about a 99% increase in the ability of an adenoviral vector comprising the chimeric hexon protein to cause a visible cytopathic effect (c.p.e.) in cells such as A549 cells or COS-1 cells (or other such cells appropriate for a neutralization assay) in the presence of the neutralizing antibody as compared to an adenoviral vector comprising the wild-type hexon protein of the first adenovirus serotype against which the neutralizing antibody is directed.

[0054] A decreased ability or inability of an adenovirus chimeric hexon protein to interact with a neutralizing antibody can be shown by, for example, a reduction of inhibition (from
about 10% to about 99%) or no inhibition at all of cell infectivity by a recombinant adenoviral vector containing the chimeric hexon protein as compared to a recombinant adenoviral vector containing a wild-type hexon protein of the first adenovirus serotype (e.g., serotype 5).

[0055] The invention also provides an adenoviral vector comprising the chimeric hexon protein described herein. Adenoviruses are generally associated with benign pathologies in humans, and the 36 kilobase (kb) adenoviral genome has been extensively studied. Adenoviral vectors can be produced in high titers (e.g., about $10^{13}$ particle forming units (pfu)), and can transfer genetic material to nonreplicating, as well as replicating, cells; in contrast with, e.g., retroviral vectors, which only transfer genetic material to replicating cells. The adenoviral genome can be manipulated to carry a large amount of exogenous DNA (up to about 8 kb), and the adenoviral capsid can potentiate the transfer of even longer sequences (Curiel et al., *Hum. Gene Ther.*, 3: 147-154 (1992)). Additionally, adenoviruses generally do not integrate into the host cell chromosome, but rather are maintained as a linear episome, thus minimizing the likelihood that a recombinant adenovirus will interfere with normal cell function. In addition to being a superior vehicle for transferring genetic material to a wide variety of cell types, adenoviral vectors represent a safe choice for gene transfer, which can be a particular concern for therapeutic applications.

[0056] Adenovirus from various origins, subtypes, or mixture of subtypes can be used as the source of the viral genome for the adenoviral vector. Non-human adenovirus (e.g., simian, avian, canine, ovine, or bovine adenoviruses) can be used to generate the adenoviral vector. For example, the adenoviral vector can be based on a simian adenovirus, including both new world and old world monkeys (see, e.g., *Virus Taxonomy: VHIth Report of the International Committee on Taxonomy of Viruses* (2005)). The phylogeny of adenoviruses that infect primates is disclosed in, e.g., Roy et al., *PLoS Pathog.*, 5(7): e100050. doi:10.1371/journal.ppat.1000503 (2009). For instance, a simian adenovirus can be of serotype 1, 3, 6, 7, 11, 16, 18, 19, 20, 27, 33, 38, 39, 48, 49, or 50, or any other simian adenoviral serotype. Other non-human adenoviruses which can be used in the invention include non-human primate adenoviruses that are genetically and/or phenotypically similar to group C human adenoviruses. A human adenovirus can be used as the source of the viral genome for the adenoviral vector. For instance, an adenovirus can be of subgroup A (e.g., serotypes 12, 18, and 31), subgroup B (e.g., serotypes 3, 7, 11, 14, 16, 21, 34, 35, and 50), subgroup C (e.g., serotypes 1, 2, 5, and 6), subgroup D (e.g., serotypes 8, 9, 10, 13, 15, 17,
19, 20, 22-30, 32, 33, 36-39, and 42-48), subgroup E (e.g., serotype 4), subgroup F (e.g., serotypes 40 and 41), an unclassified serogroup (e.g., serotypes 49 and 51), or any other adenoviral serotype. Adenoviral serotypes 1 through 51 (i.e., Ad1 through Ad51) are available from the American Type Culture Collection (ATCC, Manassas, VA). Preferably, in the context of the invention, the adenoviral vector is of human subgroup C, especially serotype 2 or even more desirably serotype 5. However, non-group C adenoviruses can be used to prepare adenoviral gene transfer vectors for delivery of gene products to host cells. Preferred adenoviruses used in the construction of non-group C adenoviral gene transfer vectors include Ad12 (group A), Ad7 and Ad35 (group B), Ad30 and Ad36 (group D), Ad4 (group E), and Ad41 (group F). Non-group C adenoviral vectors, methods of producing non-group C adenoviral vectors, and methods of using non-group C adenoviral vectors are disclosed in, for example, U.S. Patents 5,801,030, 5,837,511, and 5,849,561, and International Patent Application Publications WO 1997/012986 and WO 1998/053087.

[0057] The adenoviral vector can comprise portions of an adenoviral genome of two or more (e.g., a mixture of) subtypes, in addition to containing a nucleic acid sequence encoding the chimeric hexon protein as described herein, and thereby be a "chimeric" adenoviral vector. A chimeric adenoviral vector can comprise an adenoviral genome that is derived from two or more (e.g., 2, 3, 4, etc.) different adenovirus serotypes. In the context of the invention, a chimeric adenoviral vector can comprise approximately different or equal amounts of the genome of each of the two or more different adenovirus serotypes. When the chimeric adenoviral vector genome is comprised of the genomes of two different adenovirus serotypes, the chimeric adenoviral vector genome preferably comprises no more than about 99% (e.g., no more than about 95%, no more than about 85%, no more than about 80%, no more than about 75%, no more than about 60%, no more than about 65%, no more than about 50%, or no more than about 40%) of the genome of one of the adenovirus serotypes, with the remainder of the chimeric adenovirus genome being derived from the genome of the other adenovirus serotype.

[0058] The adenoviral vector can be replication-competent, conditionally replication-competent, or replication-deficient.

[0059] A replication-competent adenoviral vector can replicate in typical host cells, i.e., cells typically capable of being infected by an adenovirus. A replication-competent adenoviral vector can have one or more mutations as compared to the wild-type adenovirus (e.g., one or more deletions, insertions, and/or substitutions) in the adenoviral genome that do
not inhibit viral replication in host cells. For example, the adenoviral vector can have a partial or entire deletion of the adenoviral early region known as the E3 region, which is not essential for propagation of the adenoviral genome.

A conditionally-replicating adenoviral vector is an adenoviral vector that has been engineered to replicate under pre-determined conditions. For example, replication-essential gene functions, e.g., gene functions encoded by the adenoviral early regions, can be operably linked to an inducible, repressible, or tissue-specific transcription control sequence, e.g., promoter. In such an embodiment, replication requires the presence or absence of specific factors that interact with the transcription control sequence. Conditionally-replicating adenoviral vectors are further described in U.S. Patent 5,998,205.

A replication-deficient adenoviral vector is an adenoviral vector that requires complementation of one or more gene functions or regions of the adenoviral genome that are required for replication, as a result of, for example, a deficiency in one or more replication-essential gene function or regions, such that the adenoviral vector does not replicate in typical host cells, especially those in a human to be infected by the adenoviral vector.

A deficiency in a gene function or genomic region, as used herein, is defined as a disruption (e.g., deletion) of sufficient genetic material of the adenoviral genome to obliterate or impair the function of the gene (e.g., such that the function of the gene product is reduced by at least about 2-fold, 5-fold, 10-fold, 20-fold, 30-fold, or 50-fold) whose nucleic acid sequence was disrupted (e.g., deleted) in whole or in part. Deletion of an entire gene region often is not required for disruption of a replication-essential gene function. However, for the purpose of providing sufficient space in the adenoviral genome for one or more transgenes, removal of a majority of one or more gene regions may be desirable. While deletion of genetic material is preferred, mutation of genetic material by addition or substitution also is appropriate for disrupting gene function. Replication-essential gene functions are those gene functions that are required for adenovirus replication (e.g., propagation) and are encoded by, for example, the adenoviral early regions (e.g., the EI, E2, and E4 regions), late regions (e.g., the L1, L2, L3, L4, and L5 regions), genes involved in viral packaging (e.g., the IVa2 gene), and virus-associated RNAs (e.g., VA-RNA-1 and/or VA-RNA-2).

Preferably, the adenoviral vector is replication-deficient, such that the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of one or more regions of the adenoviral genome for propagation (e.g., to form adenoviral vector particles).
The replication-deficient adenoviral vector can be modified in any suitable manner to cause the deficiencies in the one or more replication-essential gene functions in one or more regions of the adenoviral genome for propagation. The complementation of the deficiencies in the one or more replication-essential gene functions of one or more regions of the adenoviral genome refers to the use of exogenous means to provide the deficient replication-essential gene functions. Such complementation can be effected in any suitable manner, for example, by using complementing cells and/or exogenous DNA (e.g., helper adenovirus) encoding the disrupted replication-essential gene functions.


The early regions of the adenoviral genome include the E1, E2, E3, and E4 regions. The E1 region comprises the E1A and E1B subregions, and one or more deficiencies in replication-essential gene functions in the E1 region can include one or more deficiencies in replication-essential gene functions in either or both of the E1A and E1B subregions, thereby requiring complementation of the E1A subregion and/or the E1B subregion of the adenoviral genome for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). The E2 region comprises the E2A and E2B subregions, and one or more deficiencies in replication-essential gene functions in the E2 region can include one or more deficiencies in replication-essential gene functions in either or both of the E2A and E2B subregions, thereby requiring complementation of the E2A subregion and/or the E2B subregion of the adenoviral genome for the adenoviral vector to propagate (e.g., to form adenoviral vector particles).

The E3 region does not include any replication-essential gene functions, such that a deletion of the E3 region in part or in whole does not require complementation of any gene.
functions in the E3 region for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). In the context of the invention, the E3 region is defined as the region that initiates with the open reading frame of the 12.5K protein from the E3 region of human adenovirus 5 (NCBI reference sequence AP_000218) and ends with the open reading frame that encodes the 14.7K protein from the E3 region of human adenovirus 5 (NCBI reference sequence AP_000224). The E3 region may be deleted in whole or in part, or retained in whole or in part. The size of the deletion may be tailored so as to retain an adenoviral vector whose genome closely matches the optimum genome packaging size. A larger deletion will accommodate the insertion of larger heterologous nucleic acid sequences in the adenoviral genome.

[0068] The E4 region comprises multiple open reading frames (ORFs). An adenoviral vector with a deletion of all of the open reading frames of the E4 region except ORF6, and in some cases ORF3, does not require complementation of any gene functions in the E4 region for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). Conversely, an adenoviral vector with a disruption or deletion of ORF6, and in some cases ORF3, of the E4 region (e.g., with a deficiency in a replication-essential gene function based in ORF6 and/or ORF3 of the E4 region), with or without a disruption or deletion of any of the other open reading frames of the E4 region or the native E4 promoter, polyadenylation sequence, and/or the right-side inverted terminal repeat (ITR), requires complementation of the E4 region (specifically, of ORF6 and/or ORF3 of the E4 region) for the adenoviral vector to propagate (e.g., to form adenoviral vector particles). The late regions of the adenoviral genome include the LI, L2, L3, L4, and L5 regions. The adenoviral vector also can have a mutation in the major late promoter (MLP), as discussed in International Patent Application Publication WO 2000/000628, which can render the adenoviral vector replication-deficient if desired.

[0069] The one or more regions of the adenoviral genome that contain one or more deficiencies in replication-essential gene functions desirably are one or more early regions of the adenoviral genome, i.e., the El, E2, and/or E4 regions, optionally with the deletion in part or in whole of the E3 region.

[0070] The replication-deficient adenoviral vector also can have one or more mutations as compared to the wild-type adenovirus (e.g., one or more deletions, insertions, and/or substitutions) in the adenoviral genome that do not inhibit viral replication in host cells. Thus, in addition to one or more deficiencies in replication-essential gene functions, the
adenoviral vector can be deficient in other respects that are not replication-essential. For example, the adenoviral vector can have a partial or entire deletion of the adenoviral early region known as the E3 region, which is not essential for propagation of the adenoviral genome.

[0071] In one embodiment, the adenoviral vector is replication-deficient and requires, at most, complementation of the E1 region or the E4 region of the adenoviral genome, for propagation (e.g., to form adenoviral vector particles). Thus, the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of the E1A subregion and/or the E1B region of the adenoviral genome (denoted an El-deficient adenoviral vector) or the E4 region of the adenoviral genome (denoted an E4-deficient adenoviral vector) for propagation (e.g., to form adenoviral vector particles). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E1 region of the adenoviral genome and at least one gene function of the nonessential E3 region of the adenoviral genome (denoted an E1/E3-deficient adenoviral vector). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E4 region of the adenoviral genome and at least one gene function of the nonessential E3 region of the adenoviral genome (denoted an E3/E4-deficient adenoviral vector).

[0072] In one embodiment, the adenoviral vector is replication-deficient and requires, at most, complementation of the E2 region, preferably the E2A subregion, of the adenoviral genome, for propagation (e.g., to form adenoviral vector particles). Thus, the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of the E2A subregion of the adenoviral genome (denoted an E2A-deficient adenoviral vector) for propagation (e.g., to form adenoviral vector particles). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E2A region of the adenoviral genome and at least one gene function of the nonessential E3 region of the adenoviral genome (denoted an E2A/E3-deficient adenoviral vector).

[0073] In one embodiment, the adenoviral vector is replication-deficient and requires, at most, complementation of the E1 and E4 regions of the adenoviral genome for propagation (e.g., to form adenoviral vector particles). Thus, the replication-deficient adenoviral vector requires complementation of at least one replication-essential gene function of both the E1 and E4 regions of the adenoviral genome (denoted an El/E4-deficient adenoviral vector) for
propagation (e.g., to form adenoviral vector particles). The adenoviral vector can be deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E1 region of the adenoviral genome, at least one replication-essential gene function of the E4 region of the adenoviral genome, and at least one gene function of the nonessential E3 region of the adenoviral genome (denoted an El/E3/E4-deficient adenoviral vector). The adenoviral vector preferably requires, at most, complementation of the E1 region of the adenoviral genome for propagation, and does not require complementation of any other deficiency of the adenoviral genome for propagation. More preferably, the adenoviral vector requires, at most, complementation of the E1 and E4 regions of the adenoviral genome for propagation, and does not require complementation of any other deficiency of the adenoviral genome for propagation.

[0074] The adenoviral vector, when deficient in multiple replication-essential gene functions of the adenoviral genome (e.g., an El/E4-deficient adenoviral vector), can include a spacer sequence to provide viral growth in a complementing cell line similar to that achieved by adenoviral vectors deficient in a single replication-essential gene function (e.g., an El-deficient adenoviral vector). The spacer sequence can contain any nucleotide sequence or sequences which are of a desired length, such as sequences at least about 15 base pairs (e.g., between about 15 nucleotides and about 12,000 nucleotides), preferably about 100 nucleotides to about 10,000 nucleotides, more preferably about 500 nucleotides to about 8,000 nucleotides, even more preferably about 1,500 nucleotides to about 6,000 nucleotides, and most preferably about 2,000 to about 3,000 nucleotides in length, or a range defined by any two of the foregoing values. The spacer sequence can be coding or non-coding and native or non-native with respect to the adenoviral genome, but does not restore the replication-essential function to the deficient region. The spacer also can contain an expression cassette. More preferably, the spacer comprises a polyadenylation sequence and/or a gene that is non-native with respect to the adenoviral vector. The use of a spacer in an adenoviral vector is further described in, for example, U.S. Patent 5,851,806 and International Patent Application Publication WO 1997/021826.

[0075] By removing all or part of the adenoviral genome, for example, the El, E3, and E4 regions of the adenoviral genome, the resulting adenoviral vector is able to accept inserts of heterologous nucleic acid sequences while retaining the ability to be packaged into adenoviral capsids. A heterologous nucleic acid sequence can be inserted at any position in the adenoviral genome so long as insertion in the position allows for the formation of the
adenoviral vector particle. The heterologous nucleic acid sequence preferably is positioned in the E1 region, the E3 region, or the E4 region of the adenoviral genome.

[0076] The replication-deficient adenoviral vector of the invention can be produced in complementing cell lines that provide gene functions not present in the replication-deficient adenoviral vector, but required for viral propagation, at appropriate levels in order to generate high titers of viral vector stock. Such complementing cell lines are known and include, but are not limited to, 293 cells (described in, e.g., Graham et al., J. Gen. Virol., 36: 59-72 (1977)), PER.C6 cells (described in, e.g., International Patent Application Publication WO 1997/000326, and U.S. Patents 5,994,128 and 6,033,908), and 293-ORF6 cells (described in, e.g., International Patent Application Publication WO 1995/34671 and Brough et al., J. Virol., 71: 9206-9213 (1997)). Other suitable complementing cell lines to produce the replication-deficient adenoviral vector of the invention include complementing cells that have been generated to propagate adenoviral vectors encoding transgenes whose expression inhibits viral growth in host cells (see, e.g., U.S. Patent Application Publication 2008/0233650). Additional suitable complementing cells are described in, for example, U.S. Patents 6,677,156 and 6,682,929, and International Patent Application Publication WO 2003/020879. In some instances, the cellular genome need not comprise nucleic acid sequences, the gene products of which complement for all of the deficiencies of a replication-deficient adenoviral vector. One or more replication-essential gene functions lacking in a replication-deficient adenoviral vector can be supplied by a helper virus, e.g., an adenoviral vector that supplies in trans one or more essential gene functions required for replication of the replication-deficient adenoviral vector. Alternatively, the inventive adenoviral vector can comprise a non-native replication-essential gene that complements for the one or more replication-essential gene functions lacking in the inventive replication-deficient adenoviral vector. For example, an El/E4-deficient adenoviral vector can be engineered to contain a nucleic acid sequence encoding E4 ORF 6 that is obtained or derived from a different adenovirus (e.g., an adenovirus of a different serotype than the inventive adenoviral vector, or an adenovirus of a different species than the inventive adenoviral vector).

[0077] The adenoviral vector can comprise a heterologous nucleic acid sequence other than the nucleic acid sequence encoding the chimeric hexon protein described herein. A "heterologous nucleic acid sequence" is any nucleic acid sequence (e.g., DNA, RNA, or cDNA sequence) that is not a naturally occurring nucleic acid sequence of an adenovirus in a naturally occurring position. Thus, the heterologous nucleic acid sequence can be naturally
found in an adenovirus, but located at a non-native position within the adenoviral genome and/or operably linked to a non-native promoter. The terms "non-native nucleic acid sequence," "heterologous nucleic acid sequence," and "exogenous nucleic acid sequence" are synonymous and can be used interchangeably in the context of the invention. The heterologous nucleic acid sequence preferably is DNA and preferably encodes a protein (i.e., one or more nucleic acid sequences encoding one or more proteins). The adenoviral vector can comprise one or multiple heterologous nucleic acid sequences (e.g., 1, 2, 3 or more heterologous nucleic acid sequences).

[0078] The heterologous nucleic acid sequence can encode a therapeutic protein that can be used to prophylactically or therapeutically treat a mammal for a disease, as described herein. For example, the therapeutic protein can be a cytokine. Examples of cytokines include, but are not limited to, interleukins, interferons (i.e., IFN-α, IFN-β, IFN-γ), leukemia inhibitory factor (LIF), oncostatin M (OSM), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), tumor necrosis factor-alpha (TNF-α), tumor necrosis factor-beta (TNF-β), and transforming growth factor-beta (TGF-β). The therapeutic protein also can be a protein that is toxic to a specific subset of cells. In this respect, the heterologous nucleic acid sequence can encode an apoptotic factor (e.g., Bax, Bak, Bcl-Xs, Bad, Bim, Bik, Bid, Harakiri, ICE-CED3 proteases, TRAIL, SARP-2, apoptin), an enzyme (e.g., cytosine deaminase, adenosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, and thymidine kinase), a toxin (e.g., ricin A-chain, diphtheria toxin A, pertussis toxin A subunit, E. coli enterotoxin A subunit, cholera toxin A subunit and pseudomonas toxin c-terminal), a ribozyme, or a cell cycle regulator (e.g., p27, p21, p57, p18, p73, pl9, pl5, E2F-1, E2F-2, E2F-3, pl07, pl30 and E2F-4). Other therapeutic proteins include, for example, proteins involved in the promotion or inhibition of angiogenesis, hormones, receptors (e.g., cell surface receptors), mitogens (e.g., growth factors), regulatory factors (e.g., gene regulatory proteins), immunoglobulins, neuropeptides, neurotransmitters, and antigen molecules.

[0079] In a particularly preferred embodiment, the heterologous nucleic acid sequence encodes an antigen. The antigen can be an antigen of any pathogen described herein. In a preferred embodiment, the heterologous nucleic acid sequence encodes an antigen from a Dengue virus (e.g., a pre-membrane and envelope fusion protein), a respiratory syncytial virus (RSV) (e.g., an RSV F protein), an Epstein Barr Virus (EBV), a Norovirus, or a Plasmodium parasite (e.g., a circumsporozoite protein (CSP)). Alternatively, the antigen can
be a tumor antigen. By "tumor antigen" is meant an antigen that is expressed by tumor cells but not normal cells, or an antigen that is expressed in normal cells but is overexpressed in tumor cells. Examples of suitable tumor antigens include, but are not limited to, β-catenin, BCR-ABL fusion protein, K-ras, N-ras, PTPRK, NY-ESO-1/LAGE-2, SSX-2, TRP2-INT2, CEA, gp100, kallikrein 4, prostate specific antigen (PSA), TRP-1/gp75, TRP-2, tyrosinase, EphA3, HER-2/neu, MUC1, p53, mdm-2, PSMA, RAGE-1, surviving, telomerase, and WTI. Other tumor antigens are known in the art and are described in, for example, The Peptide Database of T-Cell Defined Tumor Antigens, maintained by the Ludwig Institute for Cancer Research (cancerimmunity.org/statics/databases.htm), Van den Eynde et al., Curr. Opin. Immunol, 9: 684-93 (1997), Houghton et al., Curr. Opin. Immunol, 13: 134-140 (2001), and van der Bruggen et al., Immunol. Rev., 188: 51-64 (2002).

[0080] When the heterologous nucleic acid sequence encodes an antigen of a pathogen, the heterologous nucleic acid sequence comprises codons expressed more frequently in humans than in the pathogen. While the genetic code is generally universal across species, the choice among synonymous codons is often species-dependent. One of ordinary skill in the art would appreciate that, to achieve maximum protection against infection by a pathogen, the adenoviral vector must be capable of expressing high levels of antigens in a mammalian, preferably a human, host. In this respect, the nucleic acid sequence preferably encodes the native amino acid sequence of an antigen, but comprises codons that are expressed more frequently in mammals (e.g., humans) than in the pathogen. Changing all native pathogen codons to the most frequently used in mammals will increase expression of the antigen in a mammal (e.g., a human). Such modified nucleic acid sequences are commonly described in the art as "humanized," as "codon-optimized," or as utilizing "mammalian-preferred" or "human-preferred" codons.

[0081] In the context of the invention, a nucleic acid sequence from a pathogen is said to be "codon-optimized" if at least about 60% (e.g., at least about 70%, at least about 80%, or at least about 90%) of the wild-type codons in the nucleic acid sequence are modified to encode mammalian-preferred codons. That is, a pathogen nucleic acid sequence is codon-optimized if at least about 60% of the codons encoded therein are mammalian-preferred codons.

[0082] The adenoviral vector can comprise more than one heterologous nucleic acid sequences. Thus, the invention provides an adenoviral vector comprising one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) heterologous nucleic acid sequences. When the adenoviral vector comprises multiple (i.e., two or more) heterologous nucleic acid sequences,
each heterologous nucleic acid sequence can encode the same protein (e.g., the same antigen). Alternatively, the adenoviral vector can comprise multiple nucleic acid sequences encoding two or more different proteins (e.g., two or more different antigens).

[0083] When the adenoviral vector comprises two or more heterologous nucleic acid sequences, each of the heterologous nucleic acid sequences preferably is located in the E1 region or the E4 region of the adenoviral genome. Thus, in accordance with the invention, at least one heterologous nucleic acid sequence (e.g., one, two, three, or more heterologous nucleic acid sequences) can be located in the E1 region of the adenoviral genome, and at least one heterologous nucleic acid sequence (e.g., one, two, three, or more heterologous nucleic acid sequences) can be located in the E4 region of the adenoviral genome. In embodiments where the adenoviral vector comprises three or more heterologous nucleic acid sequences, at least one heterologous nucleic acid sequence preferably is located in the E1 region of the adenoviral genome, and at least two heterologous nucleic acid sequences preferably are located in the E4 region of the adenoviral genome. Alternatively, at least two heterologous nucleic acid sequences can be located in the E1 region of the adenoviral genome, and at least one heterologous nucleic acid sequence can be located in the E4 region of the adenoviral genome. While not preferred, all of the heterologous nucleic acid sequences can be located in either the E1 region or the E4 region of the adenoviral genome. The insertion of a nucleic acid sequence into the adenoviral genome (e.g., into the E1 region of the genome) can be facilitated by known methods, for example, by the introduction of a unique restriction site at a given position of the adenoviral genome. As set forth above, preferably all or part of the E3 region of the adenoviral vector also is deleted.

[0084] The heterologous nucleic acid sequence can be inserted into the adenoviral genome in a 3'-5' orientation, e.g., oriented such that the direction of transcription of the nucleic acid sequence is opposite that of the surrounding adjacent adenoviral genome. However, it is also appropriate for a heterologous nucleic acid sequence to be inserted in a 5'-3' orientation with respect to the direction of transcription of the surrounding adjacent adenovirus genome. In this regard, it is possible for the inventive adenoviral vector to comprise at least one heterologous nucleic acid sequence inserted into, for example, the E1 region in a 5'-3' orientation, and at least one heterologous nucleic acid sequence inserted into the E4 region in a 5'-3' orientation. Alternatively, the inventive adenoviral vector can comprise at least one heterologous nucleic acid sequence inserted into the E1 region in a 5'-3' orientation, and at least one heterologous nucleic acid sequence inserted into the E4 region
in a 3’-5’ orientation. In yet another embodiment, the inventive adenoviral vector can comprise at least one heterologous nucleic acid sequence inserted into the E1 region in a 3’-5’ orientation, and at least one heterologous nucleic acid sequence inserted into the E4 region in a 5’-3’ orientation with respect to the direction of transcription of the surrounding genome.

The adenoviral vector preferably comprises expression control sequences, such as promoters, enhancers, polyadenylation signals, protease cleavage sites, transcription terminators, internal ribosome entry sites (IRES), and the like, that provide for the expression of the heterologous nucleic acid sequence in a host cell. Exemplary expression control sequences are known in the art and are described in, for example, Goeddel, *Gene Expression Technology: Methods in Enzymology*, Vol. 185, Academic Press, San Diego, Calif. (1990). Ideally, the heterologous nucleic acid sequence is operably linked to a promoter and a polyadenylation sequence. A large number of promoters, including constitutive, inducible, and repressible promoters, from a variety of different sources are well known in the art. Representative sources of promoters include for example, virus, mammal, insect, plant, yeast, and bacteria, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3’ or 5’ direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, and the SV40 promoter. Inducible promoters include, for example, the Tet system (U.S. Patents 5,464,758 and 5,814,618), the Ecdysone inducible system (see, e.g., No et al., *Proc. Natl. Acad. Sci.*, 93: 3346-3351 (1996)), the T-REx™ system (Invitrogen, Carlsbad, CA), LACSWITCH™ System (Stratagene, San Diego, CA), and the Cre-ERT tamoxifen inducible recombinase system (Indra et al., *Nuc. Acid. Res.*, 27: 4324-4327 (1999); *Nuc. Acid. Res.*, 28: e99 (2000); U.S. Patent 7,1 12,715; and Kramer & Fussenegger, *Methods Mol. Biol.*, 308: 123-144 (2005)).

A promoter can be selected by matching its particular pattern of activity with the desired pattern and level of expression of a gene product(s). For example, the adenoviral vector can comprise two or more heterologous nucleic acid sequences that encode different antigens and are operably linked to different promoters displaying distinct expression profiles. In this regard, a first promoter can be selected to mediate an initial peak of antigen production, thereby priming the immune system against an encoded antigen. A second
promoter can be selected to drive production of the same or different antigen such that
expression peaks several days after that of the first promoter, thereby "boosting" the immune
system against the antigen. Alternatively, a hybrid promoter can be constructed which
combines the desirable aspects of multiple promoters. In as much as antigens can be toxic to
eukaryotic cells, it may be advantageous to modify the promoter to decrease activity in
complementing cell lines used to propagate the adenoviral vector.

[0087] Multiple heterologous nucleic acid sequences can be operably linked to the same
or different promoters. In a preferred embodiment of the invention, each heterologous
nucleic acid sequence is operably linked to a separate promoter. While it is preferred that
each promoter is different, one or ordinary skill in the art will appreciate the advantages of
using one particularly efficient promoter to control expression of each heterologous nucleic
acid sequence present in the adenoviral vector. Thus, each heterologous nucleic acid
sequence can be operably linked to the same promoter. Most preferably, each of the
heterologous nucleic acid sequences are operably linked to a different promoter. The
selection of an appropriate promoter for a given heterologous nucleic acid sequence will
depend upon a number of factors, including promoter strength and the position of the
heterologous nucleic acid sequence within the adenoviral genome, and can be performed
using routine methods known in the art.

[0088] To optimize protein production, preferably the heterologous nucleic acid sequence
further comprises a polyadenylation site following the coding sequence. Any suitable
polyadenylation sequence can be used, including a synthetic optimized sequence, as well as,
for example, the polyadenylation sequence of BGH (Bovine Growth Hormone), polyoma
virus, TK (Thymidine Kinase), EBV (Epstein Barr Virus), and the papillomaviruses,
including human papillomaviruses and BPV (Bovine Papilloma Virus). A preferred
polyadenylation sequence is the SV40 (Simian Virus-40) polyadenylation sequence. Also,
preferably all the proper transcription signals (and translation signals, where appropriate) are
correctly arranged such that the heterologous nucleic acid sequence is properly expressed in
the cells into which it is introduced. If desired, the heterologous nucleic acid sequence also
can incorporate splice sites (i.e., splice acceptor and splice donor sites) to facilitate mRNA
production.

[0089] If the heterologous nucleic acid sequence encodes a processed or secreted protein
or peptide, or a protein that acts intracellularly, preferably the heterologous nucleic acid
sequence further comprises the appropriate sequences for processing, secretion, intracellular
localization, and the like. The heterologous nucleic acid sequence can be operably linked to a signal sequence, which targets a protein to cellular machinery for secretion. Appropriate signal sequences include, but are not limited to, leader sequences for immunoglobulin heavy chains and cytokines (see, for example, Ladunga et al., Current Opinions in Biotechnology, 11: 13-18 (2000)). Other protein modifications can be required to secrete a protein from a host cell, which can be determined using routine laboratory techniques. Preparing expression constructs encoding antigens and signal sequences is further described in, for example, U.S. Patent 6,500,641. Methods of secreting non-secretable proteins are further described in, for example, U.S. Patent 6,472,176, and International Patent Application Publication WO 2002/048377.

In addition to the hexon protein, other coat proteins of an adenoviral vector can be manipulated to alter the binding specificity or recognition of the virus for a viral receptor on a potential host cell. Such manipulations can include deletion of regions of the fiber or penton, insertions of various native or non-native ligands into portions of the coat proteins, and the like. Manipulation of coat proteins can broaden the range of cells infected by the adenoviral vector or enable targeting of the adenoviral vector to a specific cell type.

In one embodiment, the adenoviral vector comprises a modified fiber protein. The fiber protein is "modified" in that it comprises a normative amino acid sequence, in addition to or in place of a wild-type fiber amino acid sequence of an adenovirus of the first serotype. Any suitable amino acid residue(s) of a wild-type fiber protein of the first adenovirus serotype that mediates or assists in the interaction between the fiber knob and the native cellular receptor can be modified or removed, so long as the fiber protein is able to trimerize. Similarly, amino acids can be added to the fiber knob as long as the fiber protein retains the ability to trimerize.

In one embodiment, at least a portion of the wild-type fiber protein of an adenovirus of the first serotype is removed and replaced with a corresponding portion of a wild-type fiber protein from a third adenovirus serotype. The third adenovirus serotype preferably is different than the first adenovirus serotype. Preferably, the entire wild-type fiber protein of an adenovirus of the first serotype is replaced with a fiber protein from an adenovirus of a third serotype. While the third adenovirus serotype is different than the first adenovirus serotype, the third adenovirus serotype can be the same as the second adenovirus serotype from which the second portion of the chimeric hexon protein is derived. Alternatively, the third adenovirus serotype can be different than the second adenovirus
serotype from which the second portion of the chimeric hexon protein is derived. The third adenovirus serotype can be any human or non-human (e.g., simian) adenovirus serotype described herein.

[0093] The modified fiber protein can comprise a non-native amino acid sequence that confers to the modified fiber protein the ability to bind to an immune cell more efficiently than a wild-type fiber protein from an adenovirus of the first serotype. In particular, the adenoviral vector can comprise a modified adenoviral fiber protein comprising a non-native amino acid sequence which facilitates uptake of the adenoviral vector by immune cells, preferably antigen presenting cells, such as dendritic cells, monocytes, and macrophages. In another embodiment, the adenoviral vector can comprise a modified fiber protein comprising an amino acid sequence (e.g., a non-native amino acid sequence) comprising an RGD motif, which increases transduction efficiency of the adenoviral vector into dendritic cells. The RGD-motif, or any non-native amino acid sequence, preferably is inserted into the adenoviral fiber knob region, ideally in an exposed loop of the adenoviral knob, such as the HI loop. A non-native amino acid sequence also can be appended to the C-terminus of the adenoviral fiber protein, optionally via a spacer sequence.


[0095] The invention also provides a method of treating a disease in a mammal which comprises administering a composition comprising the adenoviral vector described herein to the mammal, whereby the disease is treated in the mammal. The adenoviral vector described herein can be used to treat any one of a number of diseases, such as, for example, cancer, genetic disorders, or pathogenic infections (e.g., such as by Dengue virus, respiratory syncytial virus (RSV), HIV, *P. falciparum*, and herpes simplex virus 2 (HSV-2)). Therefore, the adenoviral vector desirably is administered to a mammal in a physiologically acceptable (e.g., pharmaceutically acceptable) composition, which comprises a carrier, preferably a
physiologically (e.g., pharmaceutically) acceptable carrier, and the adenoviral vector. Any suitable carrier can be used within the context of the invention, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition is to be administered and the particular method used to administer the composition. Ideally, in the context of adenoviral vectors, the pharmaceutical composition preferably is free of replication-competent adenovirus. The pharmaceutical composition can optionally be sterile.

[0096] Suitable formulations for the composition include aqueous and non-aqueous isotonic sterile solutions, which can contain anti-oxidants, buffers, and bacteriostats, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets. Preferably, the carrier is a buffered saline solution. More preferably, the adenoviral vector is administered in a composition formulated to protect the adenoviral vector from damage prior to administration. For example, the composition can be formulated to reduce loss of the adenoviral vector on devices used to prepare, store, or administer the adenoviral vector, such as glassware, syringes, or needles. The composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the adenoviral vector. To this end, the composition preferably comprises a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. Use of such a composition will extend the shelf life of the adenoviral vector, facilitate administration, and increase the efficiency of the inventive method. Formulations for adenoviral vector-containing compositions are further described in, for example, U.S. Patent 6,225,289, U.S. Patent 6,514,943, and International Patent Application Publication WO 2000/034444.

[0097] The composition also can be formulated to enhance transduction efficiency. In addition, one of ordinary skill in the art will appreciate that the adenoviral vector can be present in a composition with other therapeutic or biologically-active agents. For example, factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation associated with in vivo administration of
the adenoviral vector. As discussed herein, immune system stimulators or adjuvants, e.g., interleukins, lipopolysaccharide, or double-stranded RNA, can be administered to enhance or modify any immune response to an antigen encoded by the adenoviral vector. Antibiotics, i.e., microbicides and fungicides, can be present to treat existing infection and/or reduce the risk of future infection, such as infection associated with gene transfer procedures.

In the method of the invention, the adenoviral vector preferably is administered to a mammal (e.g., a mouse, rat, rabbit, non-human primate, or a human), wherein the heterologous nucleic acid sequence is expressed in the mammal. In embodiments where multiple adenoviral vectors are administered to a mammal, the adenoviral vectors can be separately formulated and administered simultaneously or sequentially in any order. Alternatively, the adenoviral vectors can be part of the same pharmaceutical composition.

When the heterologous nucleic acid sequence encodes an antigen of a pathogen, the heterologous nucleic acid sequence is expressed in the mammal to induce an immune response against the antigen in the mammal. The immune response can be a humoral immune response, a cell-mediated immune response, or, desirably, a combination of humoral and cell-mediated immunity. Ideally, the immune response provides protection upon subsequent challenge with the pathogen. However, protective immunity is not required in the context of the invention. The inventive method further can be used for antibody production and harvesting in non-human mammals (e.g., rabbits or mice).

To enhance the immune response generated against an antigen, the composition also can comprise an immune stimulator, or a nucleic acid sequence that encodes an immune stimulator. Immune stimulators also are referred to in the art as "adjuvants," and include, for example, cytokines, chemokines, or chaperones. Cytokines include, for example, Macrophage Colony Stimulating Factor (e.g., GM-CSF), Interferon Alpha (IFN-a), Interferon Beta (IFN-β), Interferon Gamma (IFN-γ), interleukins (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-16, and IL-18), the TNF family of proteins, Intercellular Adhesion Molecule-1 (ICAM-1), Lymphocyte Function-Associated antigen-3 (LFA-3), B7-1, B7-2, FMS-related tyrosine kinase 3 ligand, (Flt3L), vasoactive intestinal peptide (VIP), and CD40 ligand. Chemokines include, for example, B Cell-Attracting chemokine-1 (BCA-1), Fractalkine, Melanoma Growth Stimulatory Activity protein (MGSA), Hemofiltrate CC chemokine 1 (HCC-1), Interleukin 8 (IL-8), Interferon-stimulated T-cell alpha chemoattractant (I-TAC), Lymphotactin, Monocyte Chemotactic Protein 1 (MCP-1), Monocyte Chemotactic Protein 3 (MCP-3), Monocyte Chemotactic Protein 4 (CP-4),
Macrophage-Derived Chemokine (MDC), a macrophage inflammatory protein (MIP), Platelet Factor 4 (PF4), RANTES, BRAK, eotaxin, exodus 1-3, and the like. Chaperones include, for example, the heat shock proteins Hsp70, Hsc70, and Hsp40.

Any route of administration can be used to deliver the composition to the mammal. Indeed, although more than one route can be used to administer the composition, a particular route can provide a more immediate and more effective reaction than another route. Preferably, the composition is administered via intramuscular injection or intranasal administration. The composition also can be applied or instilled into body cavities, absorbed through the skin (e.g., via a transdermal patch), inhaled, ingested, topically applied to tissue, or administered parenterally via, for instance, intravenous, peritoneal, or intraarterial administration.

The adenoviral vector can be administered in or on a device that allows controlled or sustained release, such as a sponge, biocompatible meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. Patent 5,443,505) and devices (see, e.g., U.S. Patent 4,863,457), such as an implantable device, e.g., a mechanical reservoir or an implant or a device comprised of a polymeric composition, are particularly useful for administration of the adenoviral vector. The adenoviral vector also can be administered in the form of sustained-release formulations (see, e.g., U.S. Patent 5,378,475) comprising, for example, gel foam, hyaluronic acid, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), and/or a polylactic-glycolic acid.

The dose of adenoviral vector administered to the mammal will depend on a number of factors, including the disease to be treated, the size of a target tissue, the extent of any side-effects, the particular route of administration, and the like. The dose ideally comprises a "therapeutically effective amount" of adenoviral vector, i.e., a dose of adenoviral vector which induces a therapeutic or prophylactic effect in a mammal. As used herein, the terms "treatment," "treating," and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. Preferably, the effect is therapeutic, i.e., the effect partially or completely cures a disease and/or adverse symptom attributable to the disease. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result.

Alternatively, the pharmacologic and/or physiologic effect may be prophylactic, i.e., the effect completely or partially prevents a disease or symptom thereof. In this respect, the inventive method comprises administering a "prophylactically effective amount" of the
adenoviral vector. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired prophylactic result (e.g., prevention of disease onset).

[0105] In a one embodiment, the administration of the adenoviral vector provokes a desired immune response against an antigen of a pathogen in the mammal. The desired immune response can entail production of antibodies, protection upon subsequent challenge, immune tolerance, immune cell activation, and the like. Preferably, the desired immune response results in sufficient immunity for the recipient for a desired period of time such that subsequent infection with a pathogen does not result in illness.

[0106] Desirably, a single dose of adenoviral vector comprises about 1x10^5 or more particles (which also are referred to as particle units (pu)) of the adenoviral vector, e.g., about 1x10^6 or more particles, about 1x10^7 or more particles, about 1x10^8 or more particles, about 1x10^9 or more particles, or about 1x10^10 or more particles of the adenoviral vector. Alternatively, or in addition, a single dose of adenoviral vector comprises about 1x10^14 particles or less of the adenoviral vector, e.g., about 1x10^13 particles or less, about 1x10^12 particles or less, about 1x10^11 particles or less, about 1x10^10 particles or less, or about 1x10^9 particles or less of the adenoviral vector. Thus, a single dose of adenoviral vector can comprise a quantity of particles of the adenoviral vector in a range defined by any two of the aforementioned values. For example, a single dose of adenoviral vector can comprise 1x10^5-1x10^9 pu, 1x10^6-1x10^12 pu, 1x10^7-1x10^11 pu, 1x10^8-1x10^12 pu, 1x10^9-1x10^11 pu, 1x10^10-1x10^11 pu, or 1x10^11 pu of the adenoviral vector.

[0107] In embodiments where the adenoviral vector is administered to induce an immune response against an antigen of a pathogen in a mammal, administering the composition containing the adenoviral vector (or adenoviral vectors) can be one component of a multistep regimen for inducing an immune response against an antigen of a pathogen in a mammal. In particular, the inventive method can represent one arm of a prime and boost immunization regimen. In this respect, the method comprises administering to the mammal a boosting composition after administering the composition comprising the inventive adenoviral vector to the mammal. In this embodiment, therefore, the immune response is "primed" upon
administration of the composition containing the inventive adenoviral vector, and is "boosted" upon administration of the boosting composition. Alternatively, the inventive method comprises administering to the mammal a priming composition to the mammal prior to administering the composition comprising the inventive adenoviral vector to the mammal. In this embodiment, therefore, the immune response is "primed" upon administration of the priming composition, and is "boosted" upon administration of the composition containing the inventive adenoviral vector.

[0108] Each of the priming composition and the boosting composition desirably comprises a gene transfer vector that comprises a nucleic acid sequence encoding an antigen of a pathogen. While at least one of the priming composition and the boosting composition comprises at least one inventive adenoviral vector encoding an antigen of a pathogen, any gene transfer vector can be employed, including viral and non-viral gene transfer vectors, in addition to the inventive adenoviral vector. Examples of suitable viral gene transfer vectors include, but are not limited to, retroviral vectors, adeno-associated virus vectors, vaccinia virus vectors, herpesvirus vectors, parainfluenza-RSV chimeric vectors (PIV-RSV), and adenoviral vectors. Examples of suitable non-viral vectors include, but are not limited to, plasmids, liposomes, and molecular conjugates (e.g., transferrin). Preferably, each of the priming composition and the boosting composition comprises a plasmid or an adenoviral vector. Alternatively, an immune response can be primed or boosted by administration of a pathogen protein itself (e.g., an antigenic Dengue virus protein) with or without a suitable adjuvant (e.g., alum, QS-21, insulin-derived adjuvant, etc.), a killed virus, a live-attenuated virus particle, a virus-like particle, and the like. When the priming composition and/or the boosting composition comprises an adenoviral vector, the adenoviral vector can be derived from any human or non-human animal as described herein. In a preferred embodiment, the priming composition and/or the boosting composition comprises a human adenoviral vector based on, e.g., human serotypes 5, 28, or 35, or a simian adenoviral vector based on, e.g., serotypes 7, 11, 16, or 38. For example, a priming composition containing a human serotype 28 adenoviral vector can be administered to a human, followed by administration of a boosting composition containing the inventive adenoviral vector. Alternatively, a priming composition containing the inventive adenoviral vector can be administered to a human, followed by administration of a boosting composition containing a human serotype 28 adenoviral vector. In another embodiment, a priming composition containing the inventive adenoviral vector described herein can be administered to a human, followed by a second
administration of the same composition or a different composition that is in accordance with the invention. One of ordinary skill in the art will appreciate that any combination of adenoviral vectors encoding one or more antigens of a pathogen can be employed as the priming or boosting composition in conjunction with a composition comprising the adenoviral vector of the invention.

[0109] The antigen of the pathogen encoded by the nucleic acid sequence of a gene transfer vector, other than the inventive adenoviral vector, of the priming composition and/or the boosting composition can be the same as one of the antigens encoded by the inventive adenoviral vector. Alternatively, the antigen of the pathogen encoded by the nucleic acid sequence of a gene transfer vector, other than the inventive adenoviral vector, of the priming composition and/or the boosting composition can be different from the antigen(s) encoded by the inventive adenoviral vector. In one embodiment, the gene transfer vector of the priming composition and/or the boosting composition comprises multiple (i.e., two or more) nucleic acid sequences encoding the same antigen. In another embodiment, the gene transfer vector of the priming composition and/or the boosting composition can comprise multiple nucleic acid sequences encoding two or more different antigens.

[0110] Administration of the priming composition and the boosting composition can be separated by any suitable timeframe, e.g., 1 week or more, 2 weeks or more, 4 weeks or more, 8 weeks or more, 12 weeks or more, 16 weeks or more, 24 weeks or more, 52 weeks or more, or a range defined by any two of the foregoing values. The boosting composition preferably is administered to a mammal (e.g., a human) 2 weeks or more (e.g., 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 20 weeks, 24 weeks, 28 weeks, 35 weeks, 40 weeks, 50 weeks, 52 weeks, or a range defined by any two of the foregoing values) following administration of the priming composition. More than one dose of priming composition and/or boosting composition can be provided in any suitable timeframe. The dose of the priming composition and boosting composition administered to the mammal depends on a number of factors, including the extent of any side-effects, the particular route of administration, and the like.

[0111] The following examples further illustrate the present invention and, of course, should not be construed as in any way limiting its scope.
EXAMPLE 1

[0112] This example demonstrates the construction of adenoviral vectors comprising a chimeric hexon protein.

[0113] Seven adenoviral vectors based on serotype 5 adenovirus (Ad5) were generated, each of which contained one of the following hexon proteins: wild-type Ad5 hexon with all of the HVRs derived from Ad5, an Ad5-Ad43 chimeric hexon protein with all of the HVRs derived from Ad43, an Ad5-Ad43 chimeric hexon protein with some HVRs derived from Ad5 and others derived from Ad43, and an Ad5-Ad34 chimeric hexon protein with some HVRs derived from Ad5 and other HVRs derived from Ad34 (see Figure 1). Synthesized chimeric DEI loops (from DNA2.0) were cloned as KpnI-ClaI fragments into a shuttle plasmid containing partial Ad5 hexon with a deleted DEI loop. FG1 loops were PCR amplified with long oligonucleotides using Pfu Ultra DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA) and cloned as blunt fragments into another shuttle plasmid containing partial Ad5 hexon with a deleted FG1 loop. Desired combinations of hexon DEI and FG1 loops were combined into a third shuttle plasmid. Using homologous recombination (AdFast, GenVec, Inc., Gaithersburg, MD), chimeric hexons were introduced into a recipient Ad5 El/E3-deleted backbone plasmid. These plasmids were converted into vectors using 293 cells, expanded to high titers, and purified by CsCl gradient centrifugation. The serotypes of origin of each HVR in the hexon proteins are detailed in Figure 1 and Table 1 below.

Table 1

<table>
<thead>
<tr>
<th>Vector</th>
<th>HVR1</th>
<th>HVR2</th>
<th>HVR3</th>
<th>HVR4</th>
<th>HVR5</th>
<th>HVR6</th>
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[0114] HEK 293 cells were infected with each of the above-described adenoviral vectors at a multiplicity of infection (MOI) of 10 focus forming unit (ffu)/cell. The cells were
incubated at 37 °C for 72 hours, and the recombinant vectors were harvested by subjecting the cells to three cycles of freezing and thawing. The lysates were analyzed for active vector particles by a standard focus-forming unit (ffu) assay (see, e.g., Cleghon et al., *Virology*, 197: 564-575 (1993), Brough et al., *J. Virol.*, 70: 6497 (1996), and Chen et al., *Molecular Therapy*, 16: 1986 (2008)), and the resulting data are shown in Figure 2 as mean ± SEM (n=3). The results of this experiment demonstrated that an Ad5 vector comprising a hexon protein in which HVRs in the DEI loop are replaced with one or more HVRs from a serotype 43 adenovirus exhibits a 5- to 10-fold decrease in growth when compared with an Ad5 vector comprising a wild-type hexon protein.

To better understand the growth defect in the vectors comprising the Ad5-Ad43 chimeric hexon proteins, HEK 293 cells were infected with adenoviral vectors comprising these hexon proteins at an MOI of 10 ffu/cell. The cells were incubated at 32 °C for up to 98 hours. At 24 hours, 48 hours, 72 hours, and 98 hours after infection, adenoviral vectors were harvested by subjecting the cells to three cycles of freezing and thawing. The lysates were analyzed for active vector particles by a focus-forming unit (ffu) assay, and the resulting data are shown in Figure 3 as mean ± SEM (n=2). These adenoviral vectors exhibited a 100-fold decrease in growth when compared with an Ad5 vector comprising a wild-type hexon protein.

The results of this example demonstrate that an Ad5 vector comprising a hexon protein in which HVRs in the DEI loop are replaced with four or more HVRs from a serotype 43 adenovirus have a growth defect as compared to a serotype 5 vector comprising a wild-type hexon protein.

**EXAMPLE 2**

This example demonstrates the identification of adenoviral vectors comprising chimeric hexon proteins which exhibit growth rates similar to those of adenoviral vectors comprising wild-type hexon proteins.

Four of the adenoviral vectors described in Example 1 (i.e., AdH(5-5) (SEQ ID NO: 1), AdH(43m-5), AdH(5-43), and AdH(43m-43) (SEQ ID NO: 2)) were serially passaged seven times in 293 cells. In order to determine the growth rates of the vectors from the original vector stock (passage 0 (P0)) and from passage 7 (P7), 293 cells were infected at a multiplicity of infection (MOI) of 10 ffu/cell and incubated at 32 °C or 37 °C for 72 hours. The recombinant vectors were harvested by subjecting the cells to three cycles of freezing
and thawing. The lysates were analyzed for active vector particles by a focus-forming unit (ffu) assay, and the data are shown in Figures 4A and 4B as mean ± SEM (n=3).

Serial passaging of the Ad5 adenoviral vectors carrying hexon HVR regions derived from Ad43 selected for a mutant with improved growth properties, which subsequently became the dominant genetic entity in the population. By comparing the P0 data to the P7 data, the growth properties of the adenoviral vectors comprising chimeric hexon proteins at both 32 °C and 37 °C were significantly improved and similar to the growth rates of an adenoviral vector comprising a corresponding wild-type hexon protein.

Genome sequencing of these mutants revealed a single nucleotide substitution (cytosine to thymine) at position 998 in the AdH(43m-5) chimeric hexon gene and the AdH(43m-43) chimeric hexon gene (which encodes an amino acid sequence comprising SEQ ID NO: 3), which resulted in a threonine residue being replaced by a methionine residue at amino acid position 333 (T333M) in these chimeric hexon proteins. The mutation falls in the region between the DEI and FG1 loops of the Ad5 hexon protein. The position of this mutation is equivalent to nucleotide position 1025 in the wild-type Ad5 hexon gene and amino acid position 342 (T342M) in the wild-type Ad5 hexon amino acid sequence (SEQ ID NO: 1). This substitution occurred in an amino acid residue that is completely conserved in all human and simian adenovirus species. Non-primate adenoviruses typically have a N or G residue at this position.

To determine if this mutation caused the observed increase in vector yields, this mutation was cloned into the Ad5 vectors comprising chimeric hexon proteins described above, and growth of these vectors was compared to growth of the identical vectors without the mutation. The T333M mutation, in the context of the hexon 5-43 chimera, increased vector growth in 293 cells as assessed by generation of cytopathic effect at 72 hours post infection, and increased vector yields significantly at both 32 °C (Figure 5A) and 37 °C (Figure 5B).

To determine if the T333M mutation would improve the growth of adenoviral vectors comprising other chimeric hexon proteins, the mutation was built into an Ad5 vector with all nine hexon HVR sequences derived from Ad48 (Ad5H(48-48) (SEQ ID NO: 4)). This vector without the T333M mutation was reduced substantially for growth in 293 cells. However, the presence of the T333M mutation in this vector rescued vector growth when assayed at both 32 °C and 37 °C. Incorporation of the T333M mutation into a wild-type Ad5 hexon did not increase virus yields in 293 cells.
The results of this example demonstrate that an adenoviral vector comprising a chimeric hexon protein in which an Ad5-derived portion of the hexon protein contains a T333M mutation exhibits a growth rate similar to wild-type Ad5 in 293 cells.

EXAMPLE 3

This example demonstrates that adenoviral vectors comprising a chimeric hexon protein which express a *Plasmodium* protein induce immune responses in mice that are similar to immune responses induced by an adenoviral vector comprising a wild-type hexon protein.

Ad5 vectors containing hexon HVR regions from Ad43 and Ad48 as described in Example 2, with or without the T333M mutation, were engineered to contain a nucleic acid sequence encoding the *Plasmodium yoelli* circumsporozoite protein (PyCSP). BALB/c mice (n=6/group) were injected twice with 1x10^6 pu of an Ad5 vector lacking a transgene ("Adnull351 1.1 ID") to generate high titers of anti-Ad5 neutralizing antibodies (Nab), with the injections separated by four weeks. Four weeks after the second AdNull administration, the Ad5-primed mice were immunized with an injection of one of the following PyCSP-expressing adenoviral vectors: (a) Adt.PyCSP1.10, which is an El/E3-deleted Ad5 vector that is not hexon-modified, (b) Adt.(PyCSP)H(5-43m.43), which is an El/E3-deleted Ad5 vector that contains a chimeric hexon protein in which all of the HVRs are replaced with HVRs from a serotype 43 adenovirus, and (c) Adt.(PyCSP)H(5-43m.43)sp, which is identical to Adt.(PyCSP)H(5-43m.43), except that it also contains the T333M point mutation.

In parallel, a second group of mice not previously exposed to Ad5 were immunized with a dose of one of the following PyCSP-expressing adenoviral vectors: (a) Adt.PyCSP1.10, (b) Adt.(PyCSP)H(5-43m.43), (c) Adt.(PyCSP)H(5-43m.43)sp, (d) Adt.(PyCSP)H(5-48.48), which is an El/E3-deleted Ad5 vector that contains a chimeric hexon protein in which all of the HVRs are replaced with HVRs from a serotype 48 adenovirus, (e) Adt.(PyCSP)H(5-48.48)sp, which is identical to Adt.(PyCSP)H(5-48.48), except that it also contains the T333M point mutation, (f) Adt.PyCSP.1 ID is an El/E3/E4-deleted Ad5 vector that is not hexon-modified, and (g) Adt.(PyCSP)H(5-43m.43).1 ID, which is an El/E3/E4-deleted Ad5 vector that contains a chimeric hexon protein in which all of the HVRs are replaced with HVRs from a serotype 43 adenovirus. Two weeks following these administrations, T-cell responses were assessed by intracellular cytokine staining (ICS) of individual mice splenocytes. Targets were MHC-matched A20.2J cells pulsed with synthetic
peptides representing the immunodominant CD8+ T cell epitope (PyCSP280-288), a subdominant epitope (P^CSP57-70), or a defined CD8+ T cell epitope for hemagglutinin (HA 332-340).

The resulting data is set forth in Figure 6 and shows that hexon-modified adenoviral vectors carrying the T333M mutation induced T cell responses that were comparable to the hexon-modified adenoviral vectors lacking the T333M mutation, irrespective of whether Ad5-specific neutralizing antibodies were present.

The results of this example demonstrate that hexon-modified adenoviral vectors carrying the T333M mutation induced T cell responses that were similar to those induced by hexon-modified adenoviral vectors lacking the T333M mutation, and avoided preexisting adenovirus neutralizing antibodies.

EXAMPLE 4

This example demonstrates that adenoviral vectors comprising a chimeric hexon protein, with or without a chimeric fiber protein, induce immune responses in mice against a *Plasmodium* antigen that are greater than immune responses induced by Ad5 vectors.

To induce immunity against Ad5, BALB/c mice were injected intramuscularly (IM) with $1 \times 10^{10}$ pu of AdNull at 0 and 4 weeks. Four weeks after the last dose of AdNull was administered, mice were injected with $1 \times 10^8$ pu of one of the following adenoviral vectors, all of which express a transgene encoding *Plasmodium falciparum* circumsporozoite protein (PICSP): (1) an El/E3/E4-deleted Ad5 vector containing a wild-type hexon protein (AdPfCSP6.1 ID), (2) an El/E3/E4-deleted Ad5 vector containing hexon HVR regions from Ad43 and the T333M mutation as described in Example 2 (AdPfCSP6H43.1 ID), and (3) an El/E3/E4-deleted Ad5 vector containing hexon HVR regions from Ad43 and the T333M mutation as described in Example 2, and a chimeric fiber protein comprising an Ad5 tail and shaft region and a knob region from a serotype 25 simian adenovirus (SEQ ID NO: 7) (AdPfCSP6H43spF25SK.1 ID)

Three weeks after adenoviral vector administration, animal organs were harvested for T cell and antibody analysis. Serum from immunized mice was harvested three weeks after immunization to evaluate PfCSP-specific antibody responses. ELISA plates were coated overnight with a peptide composed of the repeat region of the circumsporozoite protein, CS(NANP)6C, at a concentration of 0.2 μg/ml. Plates were placed inside a humidity chamber and incubated overnight (16-20 hours) at 22 °C. Plates were washed with wash
solution (1xPBS with 0.5% tween20) and blocked in 0.5% casein blocking buffer for 1 hour at 22 °C. Plates were washed four times, and serially diluted samples were added and incubated at 22 °C for two hours. After four washings, the secondary antibody (Goat Anti-Mouse IgG(H+L), KPL, Inc., Gaithersburg, MD) was added at a dilution of 1:4000 and incubated at 22 °C for 1 hour. After extensive 4x washing, ABTS Peroxides substrate (KPL, Inc., Gaithersburg, MD) was added for development and incubated for 1 hour at 22 °C. Data were collected using SoftmaxPro4.8 (Molecular Devices, LLC, Sunnyvale, CA), and data were fit to a 4-parameter logistic curve. Mouse sera pre-validated for use in the assay was used as a positive control. Limits were set based on the pre-validation data. An OD of 1 was chosen for the cut off, as it is within the linear range of the assay.

For T cell studies, splenocytes isolated from treated mice were incubated with PfCSP-specific or control peptides as indicated in Figure 8, and antigen-specific T cell responses were evaluated by IFNy-FACS analysis.

The results of these assays are shown in Figures 7 and 8 and demonstrate that hexon-modified adenoviral vectors carrying the T333M mutation, with or without a chimeric fiber protein, induce PfCSP-specific antibody and T cell responses in mice. The results of this example also show that the antibody and T-cell responses induced by the hexon-modified adenoviral vectors are more robust in mice with high Ad5 Nab titers than an Ad5 vector lacking the hexon modification.

EXAMPLE 5

This example demonstrates that an adenoviral vector comprising a chimeric hexon protein which express a Norovirus antigen induces immune responses in mice.

Ad5 vectors containing hexon HVR regions from Ad43 containing the T333M mutation, as described in Example 2, were engineered to contain a nucleic acid sequence encoding the luciferase protein ("Adt.L"), or a norovirus antigen ("Adt.N"). The vectors were administered to BALB/c mice (n=6/group) at the dosages indicated in Figure 9. Antigen-specific antibody titers were evaluated in serum from the treated mice via ELISA. Specifically, serum from mice inoculated with the above-described vectors was harvested 21 days following vaccination and serial diluted onto an ELISA plate on which a Norovirus antigen was bound. Antibody specific to the norovirus antigen was measured by ELISA and reported as titer. The titer for each mouse is indicated as a point and defined as the log base 2
dilution at which the ELISA signal was no different than background, plus two standard
deviations of the measured background.

Example 6

This example demonstrates that adenoviral vectors comprising a chimeric hexon
protein which express an Epstein Barr Virus (EBV) antigen induce T cell responses in mice.

An Ad5 vector containing hexon HVR regions from Ad43 containing the T333M
mutation as described in Example 2 was engineered to contain a nucleic acid sequence
encoding an EBV antigen ("AdH.E"). BALB/c mice were immunized with 1x1 0⁹ pu of
AdH.E or formulation buffer sham. Splenocytes were harvested 14 days after immunization
for activation with peptide pools and individual peptides derived from the EBV antigen in 6-
hour re-stimulation assays. Specifically, splenocytes were re-stimulated with 2 μg each of six
different peptides which comprised a series of 24 predicted epitopes within the EBV antigen.
Mice injected with formulation buffer were used as negative controls. For evaluation of T
cell responses, splenocytes were fixed and stained for the CD8+ surface marker, and
intracellular levels of IFN-γ were evaluated by flow cytometry. Two experiments were
performed with five mice each, for a total of ten animals.

The results of these experiments are shown in Figure 10. Approximately 4% of
CD8+ splenocytes produced IFN-γ in response to stimulation with peptide pools 1-6, and
1.5% of CD8+ splenocytes responded to peptide pools 19-24. No significant T cell responses
were detected to peptide pools 7-12 or 13-18. Formulation buffer sham-injected animals
produced no detectable CD8+ T cell responses to either peptide pool.

The results of this example demonstrate that a hexon-modified Ad5 vector
containing the T333M mutation which encodes an EBV antigen induces robust T cell
responses in mice.
[0142] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0143] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0144] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.
CLAIMS:

1. A nucleic acid sequence encoding a chimeric adenovirus hexon protein, wherein the chimeric hexon protein comprises (a) a first portion comprising at least 10 contiguous amino acid residues of a hexon protein of a wild-type serotype 5 adenovirus, optionally with one amino acid substitution, and (b) a second portion comprising at least one hypervariable region (HVR) of a hexon protein of an adenovirus of a second adenovirus serotype, wherein the second adenovirus serotype is not serotype 5.

2. The nucleic acid sequence of claim 1, wherein the second portion of the chimeric hexon protein comprises at least one synthetic HVR.

3. The nucleic acid sequence of claim 4, wherein the synthetic HVR is an epitope of a pathogen.

4. The nucleic acid sequence of claim 6, wherein the second portion of the chimeric hexon protein comprises six HVRs of the DEI loop of a hexon protein, except that HVR1, HVR5, or both HVR1 and HVR5 are replaced with an epitope of a pathogen.

8. The adeno viral vector of any one of claims 1-7, wherein the second adenovirus serotype is serotype 43 or serotype 34.

9. The nucleic acid sequence of any one of claims 1-8, wherein the first portion of the chimeric hexon protein comprises at least 10 contiguous amino acid residues of the hexon protein of the wild-type serotype 5 adenovirus with one amino acid substitution.

10. The nucleic acid sequence of claim 9, wherein the amino acid substitution is at residue 342 of the hexon protein of the wild-type serotype 5 adenovirus.
11. The adenoviral vector of claim 10, wherein the amino acid substitution is a threonine (T) to methionine (M) substitution.

12. A nucleic acid sequence encoding a chimeric adenovirus hexon protein, wherein the chimeric hexon protein comprises (a) a first portion comprising at least 10 contiguous amino acid residues of a hexon protein of a wild-type adenovirus of a first adenovirus serotype, optionally with one amino acid substitution, and (b) a second portion comprising at least one synthetic hypervariable region (HVR) that is not present in the hexon protein of any wild-type adenovirus.

13. The nucleic acid sequence of claim 12, wherein the second portion of the chimeric hexon protein comprises at least three synthetic HVRs that are not present in the hexon protein of any wild-type adenovirus.

14. The nucleic acid sequence of claim 12 or claim 13, wherein the second portion of the chimeric hexon protein comprises nine synthetic HVRs that are not present in the hexon protein of any wild-type adenovirus.

15. The nucleic acid sequence of any one of claims 12-14, wherein the at least one synthetic HVR is a random amino acid sequence comprising between 1 and 40 amino acids.

16. The nucleic acid sequence of any one of claims 12-14, wherein the at least one synthetic HVR is an epitope of a pathogen.

17. The nucleic acid sequence of any one of claims 12-16, wherein the first adenovirus serotype is serotype 5.

18. The nucleic acid sequence of claim 17, wherein the amino acid substitution is at residue 342 of the hexon protein of the wild-type serotype 5 adenovirus.

19. The adenoviral vector of claim 18, wherein the amino acid substitution is a threonine (T) to methionine (M) substitution.

20. A chimeric adenovirus hexon protein encoded by the nucleic acid sequence of any one of claims 1-19.

22. The adenoviral vector of claim 21, wherein the adenoviral vector is replication-deficient and requires at most complementation of a deficiency in the ElA region, the ElB region, and/or the E4 region of the adenoviral genome for propagation.

23. The adenoviral vector of claim 22, wherein the adenoviral vector requires at most complementation of a deficiency in the ElA region and the ElB region of the adenoviral genome for propagation.

24. The adenoviral vector of claim 22, wherein the adenoviral vector requires at most complementation of a deficiency in the ElA region, the ElB region, and the E4 region of the adenoviral genome for propagation.

25. The adenoviral vector of any one of claims 22-24, wherein the adenoviral vector comprises a deficiency in the E3 region of the adenoviral genome.

26. The adenoviral vector of any one of claims 21-25, which comprises a heterologous nucleic acid sequence which encodes an antigen of a pathogen.

27. The adenoviral vector of claim 26, wherein the pathogen is selected from the group consisting of a respiratory syncytial virus (RSV), a Dengue virus, a Norovirus, an Epstein Barr Virus (EBV), HIV, HSV-2, or P.falciparum.