(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 April 2006 (13.04.2006)

PCT

(10) International Publication Number
WO 2006/039045 A2

(51) International Patent Classification: Not classified

(21) International Application Number: PCT/US2005/031224

(22) International Filing Date: 30 August 2005 (30.08.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/606,273 1 September 2004 (01.09.2004) US

(71) Applicants (*for all designated States except US*): **THE GOVERNMENT OF THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; National Institutes of Health, Office of Technology Transfer, 6011 Executive Boulevard, Suite 325, Rockville, MD 20852 (US). **GENVEC, INC.** [US/US]; 65 West Watkins Mill Road, Gaithersburg, MD 20878 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **NABEL, Gary, J.** [US/US]; 40 Convent Drive, Bethesda, MD 20892 (US). **CHENG, Cheng** [US/US]; 40 Convent Drive, Bethesda, MD 20892 (US). **GALL, Jason, G., D.** [US/US]; 31 Bronco Court, Germantown, MD 20874 (US). **WICKHAM, Thomas, J.** [US/US]; c/o EMD Lexigen Bedford Campus, 45A Middlesex Turnpike, Billerica, MA 01821 (US).

(74) Agents: **KOLOM, Melissa, E.** et al.; Leydig, Voit & Mayer, Ltd., Two Prudential Plaza, Suite 4900, 180 N. Stetson Ave., Chicago, IL 60601-6780 (US).

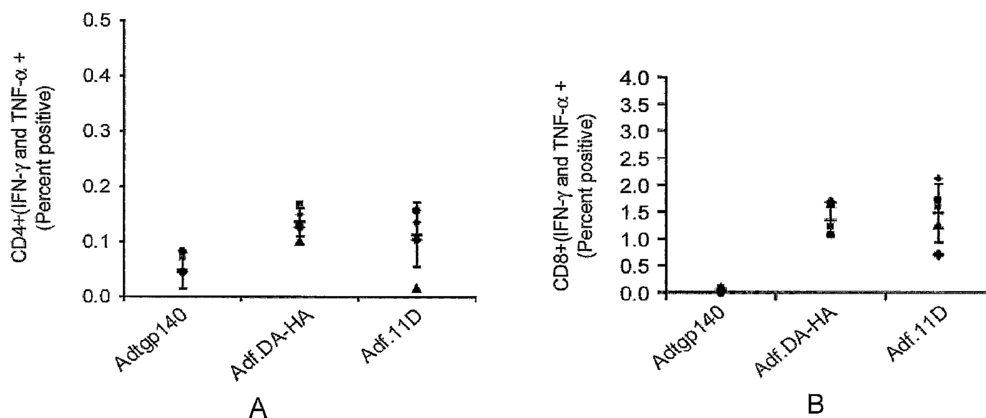
(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** METHOD OF USING ADENOVIRAL VECTORS WITH INCREASED IMMUNOGENICITY IN VIVO



(57) **Abstract:** The invention provides a method of inducing an immune response in a mammal. The method comprises administering to the mammal an adenoviral vector comprising (a) a subgroup C fiber protein wherein a native coxsackievirus and adenovirus receptor (CAR)-binding site is disrupted, (b) a subgroup C penton base protein wherein a native integrin-binding site is disrupted, and (c) a nucleic acid sequence encoding at least one antigen derived from an infectious agent other than an adenovirus which is expressed in the mammal to induce an immune response.

WO 2006/039045 A2

METHOD OF USING ADENOVIRAL VECTORS WITH INCREASED IMMUNOGENICITY IN VIVO

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0001] This invention was made in part with Government support under Cooperative Research and Development Agreement (CRADA) Number AI-1034, and amendments thereto, executed between GenVec, Inc. and the U.S. Public Health Service representing the National Institute of Allergy and Infectious Diseases. The Government may have certain rights in this invention.

FIELD OF THE INVENTION

[0002] This invention pertains to a method for inducing an immune response in a mammal comprising administering a recombinant adenoviral vector to the mammal.

BACKGROUND OF THE INVENTION

[0003] Delivery of proteins as therapeutics or for inducing an immune response to appropriate tissues in biologically-relevant amounts has been an obstacle to drug and vaccine development for decades. One solution that has proven to be a successful alternative to traditional drug delivery approaches is delivery of exogenous nucleic acid sequences for production of therapeutic factors *in vivo*. Gene transfer vectors ideally enter a wide variety of cell types, have the capacity to accept large nucleic acid sequences, are safe, and can be produced in quantities required for treating patients. Adenoviral vectors have all of these advantageous properties and are used in a variety of protocols to treat or prevent biological disorders.

[0004] Despite their advantageous properties, widespread use of adenoviral vectors is hindered, at least in part, by the fact that certain cells are not readily amenable to adenovirus-mediated gene delivery. For instance, lymphocytes, which lack the α_v integrin adenoviral receptors, are impaired in the uptake of adenoviruses (Silver et al., *Virology 165*, 377-387 (1988); Horvath et al., *J. Virology, 62*(1), 341-345 (1988)). This lack of ability to infect all cells has lead researchers to seek out ways to introduce adenovirus into cells that cannot be infected by adenovirus, e.g. due to lack of adenoviral receptors. In particular, the virus can be coupled to a DNA-polylysine complex containing a ligand (e.g., transferrin) for mammalian cells (e.g., Wagner et al., *Proc. Natl. Acad. Sci., 89*, 6099-6103 (1992); International Patent Application Publication WO 95/26412). Similarly, adenoviral fiber protein can be sterically blocked with antibodies, and tissue-specific antibodies can be chemically linked to the viral particle (Cotten et al., *Proc. Natl. Acad. Sci. USA, 89*, 6094-

6098 (1992)). In addition, adenoviral coat proteins can be modified at the genetic level to insert nucleic acid sequences encoding ligands that redirect the adenoviral vector to specific cell types (see, e.g., U.S. Patents 5,543,328 and 5,731,190).

[0005] However, these approaches are disadvantageous in that they require additional steps that covalently link large molecules, such as polylysine, receptor ligands, and antibodies, to the virus (Cotten (1992), *supra*; Wagner et al., *Proc. Natl. Acad. Sci., 89,* 6099-6103 (1992)). This adds to the size of the resultant vector as well as its cost of production. Moreover, the targeted particle complexes are not homogeneous in structure, and their efficiency is sensitive to the relative ratios of viral particles, linking molecules, and targeting molecules used. Genetic manipulation of adenoviral coat proteins has resulted in success, although somewhat limited, in selectively targeting cell types previously resistant to adenoviral infection. Thus, these approaches for expanding the repertoire of cells amenable to adenoviral-mediated gene therapy are less than optimal.

[0006] Another drawback to adenovirus-mediated gene therapy is the toxicity of the adenoviral vector in cell types typically infected by adenovirus, such as the liver (see, e.g., O'Neal et al., *Mol. Med.*, *6*, 179-195 (2000), Gallo-Penn et al., *Blood*, *7*, 107-113 (2001), and Shayakhmetov et al., *J Virol.*, *78*, 5368-5381 (2004)). The cytotoxic effect of adenovirus infection further impedes the ability of adenoviral vectors to efficiently delivery therapeutic genes to a broad range of cell types.

[0007] These disadvantages of adenoviral vector gene transfer complicate the use of these vectors as DNA vaccines. DNA vaccines employ gene transfer vectors to deliver antigen-encoding DNA to host cells. By producing antigenic proteins *in vivo*, the humoral and cell-mediated arms of the immune system are activated, thereby generating a more complete immune response against the antigen as compared to traditional vaccines wherein foreign proteins are injected into the body.

[0008] There remains a need for methods of using adenoviral vectors that are capable of infecting cells with a high efficiency and that demonstrate an alternative host cell range of infectivity to deliver nucleic acid sequences, particularly antigen-encoding nucleic acid sequences, to host cells. The invention provides such a method. These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention provides a method of inducing an immune response in a mammal, which method comprises administering to the mammal an adenoviral vector comprising (a) a subgroup C fiber protein wherein a native coxsackievirus and adenovirus receptor (CAR)-binding site is disrupted, (b) a subgroup C penton base protein wherein a native integrin-

binding site is disrupted, and (c) a nucleic acid sequence encoding at least one antigen which is expressed in the mammal to induce an immune response. Preferably, the antigen is derived from an infectious agent other than adenovirus.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1A is a graph that illustrates the percentage of GFP-specific CD4+ T lymphocytes elicited by the adenoviral vectors Adtgp140, Adf.DA-HA, and Adf.11D.

[0011] Figure 1B is a graph that illustrates the percentage of GFP-specific CD8+ T lymphocytes elicited by the adenoviral vectors Adtgp140, Adf.DA-HA, and Adf.11D.

[0012] Figure 2A is a graph that illustrates the transduction efficiencies of wild-type (wt) and mutant (mut) recombinant adenoviral vectors in murine bone marrow and dendritic cells.

[0013] Figure 2B is a graph that illustrates the dose-response of Adf.DA-HA.luc (mut ADV) in murine bone marrow cells or plasmacytoid dendritic cells.

[0014] Figure 2C is a graph that illustrates the dose-response of Adf.DA-HA.luc (mut ADV) in human bone marrow cells or plasmacytoid dendritic cells.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The invention provides materials and methods for inducing an immune response in a mammal. In particular, the invention provides adenoviral vectors suited for delivering nucleic acid sequences encoding one or more antigens to host cells and methods of using such adenoviral vectors to induce an immune response against one or more encoded antigens. The inventive method of inducing an immune response in a mammal comprises administering to the mammal an adenoviral vector comprising (a) a subgroup C fiber protein wherein a native CAR-binding site is disrupted, (b) a subgroup C penton base protein wherein a native integrin-binding site is disrupted, and (c) a nucleic acid sequence encoding at least one antigen which is expressed in the mammal to induce an immune response, wherein the antigen is derived from an infectious agent other than an adenovirus.

[0016] Adenovirus from various origins, any subtypes, or mixture of subtypes can be used as the source of the viral genome for the adenoviral vector. While non-human adenovirus (e.g., simian, avian, canine, ovine, or bovine adenoviruses) can be used to generate the adenoviral vector, a human adenovirus preferably is used as the source of the viral genome for the adenoviral vector of the inventive method. Adenovirus can be of various subgroups or serotypes. 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 are available from the American Type Culture Collection (ATCC, Manassas, VA). Preferably, in the context of the inventive method, the adenoviral vector is of human subgroup C, especially serotype 2 or even more desirably serotype 5.

[0017] The adenoviral vector can comprise a mixture of subtypes 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 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 70% (e.g., about 65%, about 50%, or 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. In one embodiment, the chimeric adenoviral vector can contain an adenoviral genome comprising a portion of a serotype 2 genome and a portion of a serotype 5 genome. For example, nucleotides 1-456 of such an adenoviral vector can be derived from a serotype 2 genome, while the remainder of the adenoviral genome can be derived from a serotype 5 genome.

[0018] The adenoviral vector of the invention can be replication competent. For example, the adenoviral vector can have a mutation (e.g., a deletion, an insertion, or a substitution) in the adenoviral genome that does not inhibit viral replication in host cells. The inventive adenoviral vector can also be conditionally-replication competent. Preferably, however, the adenoviral vector is replication-deficient in host cells.

[0019] By "replication-deficient" is meant that the adenoviral vector comprises an adenoviral genome that lacks at least one replication-essential gene function (i.e., such that the adenoviral vector does not replicate in typical host cells, especially those in a human patient that could be infected by the adenoviral vector in the course of the inventive method). A deficiency in a gene, gene function, or gene or genomic region, as used herein, is defined as a deletion of sufficient genetic material of the viral 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 deleted in whole or in part. 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 replication (e.g., propagation) and are encoded by, for example, the adenoviral early regions (e.g., the E1, E2, and E4 regions), late regions (e.g., the L1-L5 regions), genes

involved in viral packaging (e.g., the IVa2 gene), and virus-associated RNAs (e.g., VA-RNA1 and/or VA-RNA-2). More preferably, the replication-deficient adenoviral vector comprises an adenoviral genome deficient in at least one replication-essential gene function of one or more regions of the adenoviral genome. Preferably, the adenoviral vector is deficient in at least one gene function of the E1A region, the E1B region, or the E4 region of the adenoviral genome required for viral replication (denoted an E1-deficient or E4-deficient adenoviral vector). In addition to a deficiency in the E1 region, the recombinant adenovirus also can have a mutation in the major late promoter (MLP), as discussed in International Patent Application Publication WO 00/00628. Most preferably, the adenoviral vector is deficient in at least one replication-essential gene function (desirably all replication-essential gene functions) of the E1 region and at least one gene function of the nonessential E3 region (e.g., an Xba I deletion of the E3 region) (denoted an E1/E3-deficient adenoviral vector). With respect to the E1 region, the adenoviral vector can be deficient in part or all of the E1A region and part or all of the E1B region, e.g., in at least one replication-essential gene function of each of the E1A and E1B regions. When the adenoviral vector is E1-deficient, the adenoviral vector genome can comprise a deletion beginning at any nucleotide between nucleotides 335 to 375 (e.g., nucleotide 356) and ending at any nucleotide between nucleotides 3,310 to 3,350 (e.g., nucleotide 3,329) or even ending at any nucleotide between 3,490 and 3,530 (e.g., nucleotide 3,510) (based on the adenovirus serotype 5 genome). When E2A-deficient the adenoviral vector genome can comprise a deletion beginning at any nucleotide between nucleotides 22,425 to 22,465 (e.g., nucleotide 22,443) and ending at any nucleotide between nucleotides 24,010 to 24,050 (e.g., nucleotide 24,032) (based on the adenovirus serotype 5 genome). When E3-deficient, the adenoviral vector genome can comprise a deletion beginning at any nucleotide between nucleotides 28,575 to 29,615 (e.g., nucleotide 28,593) and ending at any nucleotide between nucleotides 30,450 to 30,490 (e.g., nucleotide 30,470) (based on the adenovirus serotype 5 genome). When the adenoviral vector is deficient in at least one replication-essential gene function in one region of the adenoviral genome (e.g., an E1- or E1/E3-deficient adenoviral vector), the adenoviral vector is referred to as "singly replication-deficient." A particularly preferred singly replication-deficient adenoviral vector is, for example, a replication-deficient adenoviral vector requiring, at most, complementation of the E1 region of the adenoviral genome, so as to propagate the adenoviral vector (e.g., to form adenoviral vector particles).

[0020] The adenoviral vector of the invention can be "multiply replication-deficient," meaning that the adenoviral vector is deficient in one or more replication-essential gene functions in each of two or more regions of the adenoviral genome. For example, the aforementioned E1-deficient or E1/E3-deficient adenoviral vector can be further deficient in

at least one replication-essential gene function of the E4 region (denoted an E1/E4- or E1/E3/E4-deficient adenoviral vector), and/or the E2 region (denoted an E1/E2- or E1/E2/E3-deficient adenoviral vector), preferably the E2A region (denoted an E1/E2A- or E1/E2A/E3-deficient adenoviral vector). An adenoviral vector deleted of the entire E4 region can elicit a lower host immune response. When E4-deficient, the adenoviral vector genome can comprise a deletion beginning at, for example, any nucleotide between nucleotides 32,805 to 32,845 (e.g., nucleotide 32,826) and ending at, for example, any nucleotide between nucleotides 35,540 to 35,580 (e.g., nucleotide 35,561) (based on the adenovirus serotype 5 genome), optionally in addition to deletions in the E1 region (e.g., nucleotides 356 to 3,329 or nucleotides 356 to 3,510) (based on the adenovirus serotype 5 genome) and/or deletions in the E3 region (e.g., nucleotides 28,594 to 30,469 or nucleotides 28,593 to 30,470) (based on the adenovirus serotype 5 genome).

[0021] If the adenoviral vector of the invention is deficient in a replication-essential gene function of the E2A region, the vector preferably does not comprise a complete deletion of the E2A region, which deletion preferably is less than about 230 base pairs in length. Generally, the E2A region of the adenovirus codes for a DBP (DNA binding protein), a polypeptide required for DNA replication. DBP is composed of 473 to 529 amino acids depending on the viral serotype. It is believed that DBP is an asymmetric protein that exists as a prolate ellipsoid consisting of a globular Ct with an extended Nt domain. Studies indicate that the Ct domain is responsible for DBP's ability to bind to nucleic acids, bind to zinc, and function in DNA synthesis at the level of DNA chain elongation. However, the Nt domain is believed to function in late gene expression at both transcriptional and post-transcriptional levels, is responsible for efficient nuclear localization of the protein, and also may be involved in enhancement of its own expression. Deletions in the Nt domain between amino acids 2 to 38 have indicated that this region is important for DBP function (Brough et al., *Virology*, *196*, 269-281 (1993)). While deletions in the E2A region coding for the Ct region of the DBP have no effect on viral replication, deletions in the E2A region which code for amino acids 2 to 38 of the Nt domain of the DBP impair viral replication. It is preferable that any multiply replication-deficient adenoviral vector contain this portion of the E2A region of the adenoviral genome. In particular, for example, the desired portion of the E2A region to be retained is that portion of the E2A region of the adenoviral genome which is defined by the 5' end of the E2A region, specifically positions Ad5(23816) to Ad5(24032) of the E2A region of the adenoviral genome of serotype Ad5. This portion of the adenoviral genome desirably is included in the adenoviral vector because it is not complemented in current E2A cell lines so as to provide the desired level of viral propagation.

[0022] While the above-described deletions are described with respect to an adenovirus serotype 5 genome, one of ordinary skill in the art can determine the nucleotide coordinates of the same regions of an adenovirus serotype 2 genome without undue experimentation, based on the similarity between the genomes of adenovirus serotypes 2 and 5.

[0023] In one embodiment of the inventive method, the adenoviral vector can comprise an adenoviral genome deficient in one or more replication-essential gene functions of each of the E1 and E4 regions (i.e., the adenoviral vector is an E1/E4-deficient adenoviral vector), preferably with the entire coding region of the E4 region having been deleted from the adenoviral genome. In other words, all the open reading frames (ORFs) of the E4 region have been removed. The E4 region of the adenoviral vector can retain the native E4 promoter, polyadenylation sequence, and/or the right-side inverted terminal repeat (ITR).

[0024] The adenoviral vector, when multiply replication-deficient, especially in replication-essential gene functions of the E1 and E4 regions, can include a spacer sequence to provide viral growth in a complementing cell line similar to that achieved by singly replication-deficient adenoviral vectors, particularly an E1-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 base pairs and about 12,000 base pairs), preferably about 100 base pairs to about 10,000 base pairs, more preferably about 500 base pairs to about 8,000 base pairs, even more preferably about 1,500 base pairs to about 6,000 base pairs, and most preferably about 2,000 to about 3,000 base pairs in length. The spacer element 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 element preferably is located in the E4 region of the adenoviral genome. The use of a spacer in an adenoviral vector is described in U.S. Patent 5,851,806.

[0025] It has been observed that an at least E4-deficient adenoviral vector expresses a transgene at high levels for a limited amount of time *in vivo* and that persistence of expression of a transgene in an at least E4-deficient adenoviral vector can be modulated through the action of a trans-acting factor, such as HSV ICP0, Ad pTP, CMV-IE2, CMV-IE86, HIV tat, HTLV-tax, HBV-X, AAV Rep 78, the cellular factor from the U205 osteosarcoma cell line that functions like HSV ICP0, or the cellular factor in PC12 cells that is induced by nerve growth factor, among others, as described in for example, U.S. Patents 6,225,113, 6,649,373, and 6,660,521, U.S. Patent Application Publication 2002/0031823 A1, and International Patent Application Publication WO 00/34496. In view of the above, a multiply deficient adenoviral vector (e.g., the at least E4-deficient adenoviral vector) or a second expression vector can comprise a nucleic acid sequence encoding a trans-acting

factor that modulates the persistence of expression of the nucleic acid sequence. Persistent expression of antigenic DNA can be desired when generating immune tolerance.

[0026] Desirably, the adenoviral vector requires, at most, complementation of replication-essential gene functions of the E1, E2A, and/or E4 regions of the adenoviral genome for replication (i.e., propagation). However, the adenoviral genome can be modified to disrupt one or more replication-essential gene functions as desired by the practitioner, so long as the adenoviral vector remains deficient and can be propagated using, for example, complementing cells and/or exogenous DNA (e.g., helper adenovirus) encoding the disrupted replication-essential gene functions. In this respect, the adenoviral vector can be deficient in replication-essential gene functions of only the early regions of the adenoviral genome, only the late regions of the adenoviral genome, and both the early and late regions of the adenoviral genome. Suitable replication-deficient adenoviral vectors, including multiply replication-deficient adenoviral vectors, are disclosed in U.S. Patents 5,837,511; 5,851,806; 5,994,106; 6,127,175; and 6,482,616; U.S. Patent Application Publications 2001/0043922 A1, 2002/0004040 A1, 2002/0031831 A1, 2002/0110545 A1, and 2004/0161848 A1, and International Patent Application Publications WO 94/28152, WO 95/02697, WO 95/16772, WO 95/34671, WO 96/22378, WO 97/12986, WO 97/21826, and WO 03/022311. Ideally, the replication-deficient adenoviral vector is present in a composition, e.g., a pharmaceutical composition, substantially free of replication-competent adenovirus (RCA) contamination (e.g., the pharmaceutical composition comprises less than about 1% of RCA contamination). Most desirably, the composition is RCA-free. Adenoviral vector compositions and stocks that are RCA-free are described in U.S. Patent 5,944,106, U.S. Patent Application Publication 2002/0110545 A1, and International Patent Application Publication WO 95/34671.

[0027] By removing all or part of, for example, the E1, E3, and E4 regions of the adenoviral genome, the resulting adenoviral vector is able to accept inserts of exogenous nucleic acid sequences while retaining the ability to be packaged into adenoviral capsids. The nucleic acid sequence can be positioned in the E1 region, the E3 region, or the E4 region of the adenoviral genome. Indeed, the nucleic acid sequence can be inserted anywhere in the adenoviral genome so long as the position does not prevent expression of the nucleic acid sequence or interfere with packaging of the adenoviral vector.

[0028] If the adenoviral vector is not replication-deficient, ideally the adenoviral vector is manipulated to limit replication of the vector to within a target tissue. For example, the adenoviral vector can be a conditionally-replicating adenoviral vector, which is engineered to replicate under conditions pre-determined by the practitioner. 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 this embodiment, replication requires the presence or absence of specific factors that interact with the transcription control sequence. In autoimmune disease treatment, it can be advantageous to control adenoviral vector replication in, for instance, lymph nodes, to obtain continual antigen production and control immune cell production. Conditionally-replicating adenoviral vectors are described further in U.S. Patent 5,998,205.

[0029] The invention is predicated, at least in part, on the surprising observation that adenoviral vectors, particularly subgroup C adenoviral vectors, deficient in binding to native cell surface receptors are as efficient in eliciting immune responses against encoded antigens as are adenoviral vectors retaining native binding, suggesting that these adenoviral vectors enter cells by an alternate route. Two or more of the subgroup C adenoviral coat proteins are believed to mediate attachment to cell surfaces (e.g., the fiber and penton base). Subgroup C adenovirus transduces cells via binding of the adenoviral fiber protein to the coxsackievirus and adenovirus receptor (CAR) and binding of penton proteins to integrins located on the cell surface. Subgroup C adenovirus also can bind the major histocompatability complex-I (MHC I) $\alpha 2$ domain and heparin sulfate glycosaminoglycans via the knob region and shaft region of the fiber protein, respectively (see, e.g., Hong et al., *EMBO J.*, *16*, 2294-2306 (1997), and Dechecchi et al., *J. Virol.*, *75*, 8772-8780 (2001)).

[0030] Thus, in the inventive method, native binding of adenoviral coat proteins to a cell surface receptor is interrupted. In particular, the adenoviral vector comprises a subgroup C fiber protein wherein a native coxsackievirus and adenovirus receptor (CAR)-binding site is disrupted, and a subgroup C penton base protein wherein a native integrin-binding site is disrupted. By a "subgroup C" fiber protein and penton base protein is meant that at least about 75% (e.g., about 85%, about 95%, or about 100%) of the fiber and penton base amino acid sequences are derived from a subgroup C adenovirus. Preferably, a subgroup C fiber protein and penton base protein each comprises an amino acid sequence of which at least about 90% (e.g., about 95%, about 99%, or about 100%) is derived from a subgroup C adenovirus. Most preferably, a subgroup C fiber protein and penton base protein each comprises an amino acid sequence of which at least about 100% is derived from a subgroup C adenovirus.

[0031] Any suitable technique for altering native binding to a host cell (e.g., binding to CAR) can be employed. For example, differing fiber lengths can be exploited to ablate native binding to cells. This optionally can be accomplished via the addition of a binding sequence to the penton base or fiber knob. This addition of a binding sequence can be done either directly or indirectly via a bispecific or multispecific binding sequence. In an alternative embodiment, the adenoviral fiber protein can be modified to reduce the number

of amino acids in the fiber shaft, thereby creating a "short-shafted" fiber (as described in, for example, U.S. Patent 5,962,311).

[0032] In yet another embodiment, the nucleic acid residues encoding amino acid residues associated with native substrate binding can be changed, supplemented or deleted (see, e.g., International Patent Application Publication WO 00/15823; Einfeld et al., *J. Virol., 75*(23), 11284-11291 (2001); and van Beusechem et al., *J. Virol., 76*(6), 2753-2762 (2002)) such that the adenoviral vector incorporating the mutated nucleic acid residues (or having the fiber protein encoded thereby) is less able to bind its native substrate. In this respect, the native CAR and integrin binding sites of the adenoviral vector, such as the knob domain of the adenoviral fiber protein and an Arg-Gly-Asp (RGD) sequence located in the adenoviral penton base, respectively, can be removed or disrupted. Any suitable amino acid residue(s) of a subgroup C fiber protein that mediates or assists in the interaction between the knob and CAR can be mutated 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. Suitable residues include amino acids within the exposed loops of the serotype 5 fiber knob domain, such as, for example, the AB loop, the DE loop, and the FG loop, which are further described in, for example, Roelvink et al., *Science, 286*, 1568-1571 (1999), and U.S. Patent 6,455,314. Any suitable amino acid residue(s) of a subgroup C penton base protein that mediates or assists in the interaction between the penton base and integrins can be mutated or removed. Suitable residues include, for example, one or more of the five RGD amino acid sequence motifs located in the hypervariable region of the Ad5 penton base protein (as described, for example, U.S. Patent 5,731,190). The native integrin binding sites on the subgroup C penton base protein also can be disrupted by modifying the nucleic acid sequence encoding the native RGD motif such that the native RGD amino acid sequence is conformationally inaccessible for binding to the α_v integrin receptor, such as by inserting a DNA sequence into or adjacent to the nucleic acid sequence encoding the adenoviral penton base protein. Preferably, the adenoviral vector comprises a subgroup C fiber protein and a subgroup C penton base protein that do not bind to CAR and integrins, respectively. Alternatively, the adenoviral vector comprises subgroup C fiber protein and a subgroup C penton base protein that bind to CAR and integrins, respectively, but with less affinity than the corresponding wild type subgroup C coat proteins. The adenoviral vector exhibits reduced binding to CAR and integrins if a modified adenoviral fiber protein and penton base protein binds CAR and integrins, respectively, with at least about 5-fold, 10-fold, 20-fold, 30-fold, 50-fold, or 100-fold less affinity than a non-modified adenoviral fiber protein and penton base protein of the same serotype.

[0033] Disruption of native binding of adenoviral coat proteins to a cell surface receptor can also render it less able to interact with the innate or acquired host immune system. Aside from pre-existing immunity, adenoviral vector administration induces inflammation and activates both innate and acquired immune mechanisms. Adenoviral vectors activate antigen-specific (e.g., T-cell dependent) immune responses, which limit the duration of transgene expression following an initial administration of the vector. In addition, exposure to adenoviral vectors stimulates production of neutralizing antibodies by B cells, which can preclude gene expression from subsequent doses of adenoviral vector (Wilson & Kay, *Nat. Med.*, *3*(9), 887-889 (1995)). Indeed, the effectiveness of repeated administration of the vector can be severely limited by host immunity. In addition to stimulation of humoral immunity, cell-mediated immune functions are responsible for clearance of the virus from the body. Rapid clearance of the virus is attributed to innate immune mechanisms (see, e.g., Worgall et al., *Human Gene Therapy*, *8*, 37-44 (1997)), and likely involves Kupffer cells found within the liver. Thus, an adenoviral vector comprising a subgroup C fiber protein and a subgroup C penton base protein ablated for native binding desirably is not recognized by the host immune system, thereby overcoming pre-existing immunity to Ad5 and increasing vector tolerance by the host.

[0034] The adenoviral vector also can comprise a chimeric coat protein comprising a non-native amino acid sequence that binds that binds a substrate (i.e., a ligand), such as a cellular receptor other than CAR the α_v integrin receptor. As the inventive method allows an adenoviral vector to bind, and desirably, infect host cells not naturally infected by the corresponding adenovirus that retains the ability to bind native cell surface receptors, the inventive method is particularly suited for use of "targeted" adenoviral vectors, which comprise a non-native amino acid sequence that preferentially binds a target cell, thereby further expanding the repertoire of cell types infected by the adenoviral vector. The non-native amino acid sequence of the chimeric adenoviral coat protein allows an adenoviral vector comprising the chimeric coat protein to bind and, desirably, infect host cells not naturally infected by either an adenoviral vector comprising a subgroup C fiber protein and penton base protein that retain native binding, or a corresponding adenovirus without the non-native amino acid sequence (i.e., host cells not infected by the corresponding wild-type adenovirus), to bind to host cells naturally infected by the corresponding adenovirus with greater affinity than the corresponding adenovirus without the non-native amino acid sequence, or to bind to particular target cells with greater affinity than non-target cells. A "non-native" amino acid sequence can comprise an amino acid sequence not naturally present in the adenoviral coat protein or an amino acid sequence found in the adenoviral coat but located in a non-native position within the capsid. By "preferentially binds" is meant that the non-native amino acid sequence binds a receptor, such as, for instance, $\alpha v\beta 3$

integrin, with at least about 3-fold greater affinity (e.g., at least about 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 35-fold, 45-fold, or 50-fold greater affinity) than the non-native ligand binds a different receptor, such as, for instance, $\alpha v\beta 1$ integrin.

[0035] Desirably, the adenoviral vector comprises a chimeric coat protein comprising a non-native amino acid sequence that confers to the chimeric coat protein the ability to bind to an immune cell more efficiently than a wild-type adenoviral coat protein. In particular, the adenoviral vector can comprise a chimeric 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 a preferred embodiment, the adenoviral vector comprises a chimeric fiber protein comprising an amino acid sequence (e.g., a non-native amino acid sequence) comprising an RGD motif including, but not limited to, CRGDC (SEQ ID NO: 1), CXCRGDCXC (SEQ ID NO: 2), wherein X represents any amino acid, and CDCRGDCFC (SEQ ID NO: 3), which increases transduction efficiency of an adenoviral vector into dendritic cells. The RGD-motif, or any non-native amino acid sequence ligand, 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. The spacer sequence preferably comprises between one and two-hundred amino acids, and can (but need not) have an intended function.

[0036] Where dendritic cells are the desired target cell, the non-native amino acid sequence can optionally recognize a protein typically found on dendritic cell surfaces such as adhesion proteins, chemokine receptors, complement receptors, co-stimulation proteins, cytokine receptors, high level antigen presenting molecules, homing proteins, marker proteins, receptors for antigen uptake, signaling proteins, virus receptors, etc. Examples of such potential ligand-binding sites in dendritic cells include $\alpha_v\beta_3$ integrins, $\alpha_v\beta_5$ integrins, 2A1, 7-TM receptors, CD1, CD11a, CD11b, CD11c, CD21, CD24, CD32, CD4, CD40, CD44 variants, CD46, CD49d, CD50, CD54, CD58, CD64, ASGPR, CD80, CD83, CD86, E-cadherin, integrins, M342, MHC-I, MHC-II, MIDC-8, MMR, OX62, p200-MR6, p55, S100, TNF-R, etc. Where dendritic cells are targeted, the ligand preferably recognizes the CD40 cell surface protein, such as, for example, by way of a CD-40 (bi)specific antibody fragment or by way of a domain derived from the CD40L polypeptide.

[0037] Where macrophages are the desired target, the non-native amino acid sequence optionally can recognize a protein typically found on macrophage cell surfaces, such as phosphatidylserine receptors, vitronectin receptors, integrins, adhesion receptors, receptors involved in signal transduction and/or inflammation, markers, receptors for induction of cytokines, or receptors up-regulated upon challenge by pathogens, members of the group B

scavenger receptor cysteine-rich (SRCR) superfamily, sialic acid binding receptors, members of the Fc receptor family, B7-1 and B7-2 surface molecules, lymphocyte receptors, leukocyte receptors, antigen presenting molecules, and the like. Examples of suitable macrophage surface target proteins include, but are not limited to, heparin sulfate proteoglycans, $\alpha_v\beta_3$ integrins, $\alpha_v\beta_5$ integrins, B7-1, B7-2, CD11c, CD13, CD16, CD163, CD1a, CD22, CD23, CD29, Cd32, CD33, CD36, CD44, CD45, CD49e, CD52, CD53, CD54, CD71, CD87, CD9, CD98, Ig receptors, Fc receptor proteins (e.g., subtypes of Fcα, Fcγ, Fcε, etc.), folate receptor b, HLA Class I, Sialoadhesin, siglec-5, and the toll-like receptor-2 (TLR2).

[0038] Where B-cells are the desired target, the ligand can recognize a protein typically found on B-cell surfaces, such as integrins and other adhesion molecules, complement receptors, interleukin receptors, phagocyte receptors, immunoglobulin receptors, activation markers, transferrin receptors, members of the scavenger receptor cysteine-rich (SRCR) superfamily, growth factor receptors, selectins, MHC molecules, TNF-receptors, and TNF-R associated factors. Examples of typical B-cell surface proteins include β-glycan, B cell antigen receptor (BAC), B7-2, B-cell receptor (BCR), C3d receptor, CD1, CD18, CD19, CD20, CD21, CD22, CD23, CD35, CD40, CD5, CD6, CD69, CD69, CD71, CD79a/CD79b dimer, CD95, endoglin, Fas antigen, human Ig receptors, Fc receptor proteins (e.g., subtypes of Fca, Fcg, Fcε, etc.), IgM, gp200-MR6, Growth Hormone Receptor (GH-R), ICAM-1, ILT2, CD85, MHC class I and II molecules, transforming growth factor receptor (TGF-R), $\alpha_4\beta_7$ integrin, and $\alpha_v\beta_3$ integrin.

[0039] In another embodiment, the adenoviral vector can comprise a chimeric virus coat protein that is not selective for a specific type of eukaryotic cell. The chimeric coat protein differs from a wild-type coat protein by an insertion of a non-native amino acid sequence into or in place of an internal coat protein sequence, or attachment of a non-native amino acid sequence to the N- or C- terminus of the coat protein. For example, a ligand comprising about five to about nine lysine residues (preferably seven lysine residues) is attached to the C-terminus of the adenoviral fiber protein via a non-functional spacer sequence. In this embodiment, the chimeric virus coat protein efficiently binds to a broader range of eukaryotic cells than a wild-type virus coat, such as described in U.S. Patent 6,465,253 and International Patent Application Publication WO 97/20051. Such an adenoviral vector can ensure widespread production of the antigen.

[0040] The ability of an adenoviral vector to recognize a potential host cell can be modulated without genetic manipulation of the coat protein, i.e., through use of a bi-specific molecule. For instance, complexing an adenovirus with a bispecific molecule comprising a penton base-binding domain and a domain that selectively binds a particular cell surface binding site enables the targeting of the adenoviral vector to a particular cell type.

Likewise, an antigen can be conjugated to the surface of the adenoviral particle through non-genetic means.

[0041] A non-native amino acid sequence can be conjugated to any of the adenoviral coat proteins to form a chimeric adenoviral coat protein. Therefore, for example, a non-native amino acid sequence can be conjugated to, inserted into, or attached to a fiber protein, a penton base protein, a hexon protein, proteins IX, VI, or IIIa, etc. The sequences of such proteins, and methods for employing them in recombinant proteins, are well known in the art (see, e.g., U.S. Patents 5,543,328; 5,559,099; 5,712,136; 5,731,190; 5,756,086; 5,770,442; 5,846,782; 5,962,311; 5,965,541; 5,846,782; 6,057,155; 6,127,525; 6,153,435; 6,329,190; 6,455,314; 6,465,253; 6,576,456; 6,649,407; 6,740,525, and International Patent Application Publications WO 96/07734, WO 96/26281, WO 97/20051, WO 98/07877, WO 98/07865, WO 98/40509, WO 98/54346, WO 00/15823, WO 01/58940, and WO 01/92549). The chimeric adenoviral coat protein can be generated using standard recombinant DNA techniques known in the art. Preferably, the nucleic acid sequence encoding the chimeric adenoviral coat protein is located within the adenoviral genome and is operably linked to a promoter that regulates expression of the coat protein in a wild-type adenovirus. Alternatively, the nucleic acid sequence encoding the chimeric adenoviral coat protein is located within the adenoviral genome and is part of an expression cassette which comprises genetic elements required for efficient expression of the chimeric coat protein.

[0042] The coat protein portion of the chimeric adenovirus coat protein can be a full-length adenoviral coat protein to which the ligand domain is appended, or it can be truncated, e.g., internally or at the C- and/or N- terminus. However modified (including the presence of the non-native amino acid), the chimeric coat protein preferably is able to incorporate into an adenoviral capsid. Where the non-native amino acid sequence is attached to the fiber protein, preferably it does not disturb the interaction between viral proteins or fiber monomers. Thus, the non-native amino acid sequence preferably is not itself an oligomerization domain, as such can adversely interact with the trimerization domain of the adenovirus fiber. Preferably the non-native amino acid sequence is added to the virion protein, and is incorporated in such a manner as to be readily exposed to a substrate, cell surface-receptor, or immune cell (e.g., at the N- or C- terminus of the adenoviral protein, attached to a residue facing a substrate, positioned on a peptide spacer, etc.) to maximally expose the non-native amino acid sequence. Ideally, the non-native amino acid sequence is incorporated into an adenoviral fiber protein at the C-terminus of the fiber protein (and attached via a spacer) or incorporated into an exposed loop (e.g., the HI loop) of the fiber to create a chimeric coat protein. Where the non-native amino acid sequence is attached to or replaces a portion of the penton base, preferably it is within the hypervariable regions to ensure that it contacts the substrate. Where the non-native amino

acid sequence is attached to the hexon, preferably it is within a hypervariable region (Miksza et al., *J. Virol.*, *70*(3), 1836-44 (1996)). Where the non-native amino acid is attached to or replaces a portion of pIX, preferably it is within the C-terminus of pIX. Use of a spacer sequence to extend the non-native amino acid sequence away from the surface of the adenoviral particle can be advantageous in that the non-native amino acid sequence can be more available for binding to a receptor, and any steric interactions between the non-native amino acid sequence and the adenoviral fiber monomers can be reduced.

[0043] Binding affinity of a non-native amino acid sequence to a cellular receptor can be determined by any suitable assay, a variety of which assays are known, and are useful in selecting a non-native amino acid sequence for incorporating into an adenoviral coat protein. Desirably, the transduction levels of host cells are utilized in determining relative binding efficiency. Thus, for example, host cells displaying $\alpha v\beta 3$ integrin on the cell surface (e.g., MDAMB435 cells) can be exposed to an adenoviral vector comprising the chimeric coat protein and the corresponding adenovirus without the non-native amino acid sequence, and then transduction efficiencies can be compared to determine relative binding affinity. Similarly, both host cells displaying $\alpha v\beta 3$ integrin on the cell surface (e.g., MDAMB435 cells) and host cells displaying predominantly $\alpha v\beta 1$ on the cell surface (e.g., 293 cells) can be exposed to the adenoviral vectors comprising the chimeric coat protein, and then transduction efficiencies can be compared to determine binding affinity.

[0044] In other embodiments (e.g., to facilitate purification or propagation within a specific engineered cell type), a non-native amino acid (e.g., ligand) can bind a compound other than a cell-surface protein. Thus, the ligand can bind blood- and/or lymph-borne proteins (e.g., albumin), synthetic peptide sequences such as polyamino acids (e.g., polylysine, polyhistidine, etc.), artificial peptide sequences (e.g., FLAG), and RGD peptide fragments (Pasqualini et al., *J. Cell. Biol.*, *130*, 1189 (1995)). A ligand can even bind non-peptide substrates, such as plastic (e.g., Adey et al., *Gene*, *156*, 27 (1995)), biotin (Saggio et al., *Biochem. J.*, *293*, 613 (1993)), a DNA sequence (Cheng et al., *Gene*, *171*, 1 (1996); Krook et al., *Biochem. Biophys., Res. Commun.*, *204*, 849 (1994)), streptavidin (Geibel et al., *Biochemistry*, *34*, 15430 (1995); Katz, *Biochemistry*, *34*, 15421 (1995)), nitrostreptavidin (Balass et al., *Anal. Biochem.*, *243*, 264 (1996)), heparin (Wickham et al., *Nature Biotechnol.*, *14*, 1570-73 (1996)), or other potential substrates.

[0045] Suitable modifications to an adenoviral vector are described in U.S. Patents 5,543,328; 5,559,099; 5,712,136; 5,731,190; 5,756,086; 5,770,442; 5,846,782; 5,871,727; 5,885,808; 5,922,315; 5,962,311; 5,965,541; 6,057,155; 6,127,525; 6,153,435; 6,329,190; 6,455,314; 6,465,253; 6,576,456; 6,649,407; and 6,740,525; U.S. Patent Application Publications 2001/0047081 A1, 2002/0099024 A1, 2002/0151027 A1, 2003/0022355 A1, and 2003/0099619 A1, and International Patent Applications WO 96/07734, WO 96/26281,

WO 97/20051, WO 98/07865, WO 98/07877, WO 98/40509, WO 98/54346, WO 00/15823, WO 01/58940, and WO 01/92549.

[0046] The adenoviral vector of the invention comprises a nucleic acid sequence encoding an antigen which is expressed in the mammal to induce an immune response. An "antigen" is a molecule that triggers an immune response in a mammal. An "immune response" can entail, for example, antibody production and/or the activation of immune effector cells. An antigen in the context of the invention can comprise any subunit 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. The antigen also can be a self antigen, i.e., an autologous protein which the body reacts to as if it is a foreign invader.

[0047] The antigen optionally can be derived from, obtained from, or based upon any suitable infectious agent. By "infectious agent" is meant any microorganism that causes disease in an animal, preferably a human. An antigen is "derived" from a source when it is isolated from a source and may be modified in any suitable manner (e.g., by deletion, substitution (mutation), or other modification to the sequence). An antigen is "obtained" from a source when it is isolated from that source. An antigen is "based upon" a source when the antigen is highly homologous to the source antigen, but obtained through synthetic procedures (e.g., polynucleotide synthesis, directed evolution, etc.). Suitable infectious agents include, for example, viruses, bacteria, fungi, and protozoa and portions of gene products thereof. Most preferably, the antigen is derived from an infectious agent other than an adenovirus. The nucleic acid sequence encoding the antigen is not limited to a type of nucleic acid sequence or any particular origin. The nucleic acid sequence optionally can be recombinant DNA, can be genomic DNA, or can be obtained from a DNA library of potential antigenic epitopes.

[0048] In one embodiment, the antigen is a viral antigen. The viral antigen 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*, *Capillovirus*, *Carlavirus*, *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), *Herpesviridae* (e.g., Human herpesvirus 1, 3, 4, 5, and 6, and Cytomegalovirus), *Hypoviridae*, *Iridoviridae*, *Leviviridae*, *Lipothrixviridae*, *Microviridae*, *Orthomyxoviridae* (e.g., Influenzavirus A and B), *Papovaviridae*,

Paramyxoviridae (e.g., measles, mumps, and human respiratory syncytial virus), *Parvoviridae*, *Picornaviridae* (e.g., enterovirus, poliovirus, rhinovirus, hepatovirus, and aphthovirus), *Plasmodiidae* (e.g., *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale,* and *Plasmodium malariae*), *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*. Preferably, at least one antigen of the inventive method is a retroviral antigen. The retroviral antigen can be, for example, an HIV antigen, such as 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. Also preferably, at least one antigen encoded by the adenoviral vector is a coronavirus antigen, such as a SARS virus antigen. Suitable SARS virus antigens for the inventive method include, for example, all or part of the E protein, the M protein, and the spike protein of the SARS virus. 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.

[0049] The antigen can be a parasite antigen such as, but not limited to, a *Sporozoan* antigen. For example, the nucleic acid sequence can encode a *Plasmodian* antigen, such as all or part of a Circumsporozoite protein, a Sporozoite surface protein, a liver stage antigen, an apical membrane associated protein, or a Merozoite surface protein.

[0050] Alternatively or in addition, at least one antigen encoded by the adenoviral vector is a bacterial antigen. The antigen can originate from any bacterium including, but not limited to, *Actinomyces*, *Anabaena*, *Bacillus*, *Bacteroides*, *Bdellovibrio*, *Caulobacter*, *Chlamydia*, *Chlorobium*, *Chromatium*, *Clostridium*, *Cytophaga*, *Deinococcus*, *Escherichia*, *Halobacterium*, *Heliobacter*, *Hyphomicrobium*, *Methanobacterium*, *Micrococcus*, *Myobacterium*, *Mycoplasma*, *Myxococcus*, *Neisseria*, *Nitrobacter*, *Oscillatoria*, *Prochloron*, *Proteus*, *Pseudomonas*, *Phodospirillum*, *Rickettsia*, *Salmonella*, *Shigella*, *Spirillum*, *Spirochaeta*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Sulfolobus*, *Thermoplasma*, *Thiobacillus*, and *Treponema*. In a preferred embodiment, at least one antigen encoded by the nucleic acid sequence is a *Pseudomonas* antigen or a *Heliobacter* antigen.

[0051] It will be appreciated that an entire, intact viral or bacterial protein is not required to produce an immune response. Indeed, most antigenic epitopes are relatively small in size and, therefore, protein fragments can be sufficient for exposure to the immune system of the mammal. In addition, a fusion protein can be generated between two or more antigenic epitopes of one or more antigens. For example, all or part of HIV envelope, gp120 or gp 160, can be fused to all or part of the HIV pol protein to generate a more complete immune response against the HIV pathogen compared to that generated by a

single epitope. Delivery of fusion proteins via adenoviral vector to a mammal allows exposure of an immune system to multiple antigens and, accordingly, enables a single vaccine composition to provide immunity against multiple pathogens or multiple epitopes of a single pathogen.

[0052] The nucleic acid encoding the antigen is desirably present as part of an expression cassette, i.e., a particular nucleotide sequence that possesses functions which facilitate subcloning and recovery of a nucleic acid sequence (e.g., one or more restriction sites) or expression of a nucleic acid sequence (e.g., polyadenylation or splice sites). The nucleic acid preferably is located in the E1 region (e.g., replaces the E1 region in whole or in part) or the E4 region of the adenoviral genome. For example, the E1 region can be replaced by a promoter-variable expression cassette comprising a nucleic acid encoding an antigen. The expression cassette optionally can be inserted in a 3'-5' orientation, e.g., oriented such that the direction of transcription of the expression cassette is opposite that of the surrounding adjacent adenoviral genome. However, it is also appropriate for the expression cassette to be inserted in a 5'-3' orientation with respect to the direction of transcription of the surrounding genome. In addition to the expression cassette comprising the nucleic acid encoding an antigen, the adenoviral vector can comprise other expression cassettes containing other exogenous nucleic acids, which cassettes can replace any of the deleted regions of the adenoviral genome. The insertion of an expression cassette 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.

[0053] Preferably, the nucleic acid is operably linked to (i.e., under the transcriptional control of) one or more promoter and/or enhancer elements, for example, as part of a promoter-variable expression cassette. Techniques for operably linking sequences together are well known in the art. A "promoter" is a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis. A nucleic acid sequence is "operably linked" to a promoter when the promoter is capable of directing transcription of that nucleic acid sequence. A promoter can be native or non-native to the nucleic acid sequence to which it is operably linked.

[0054] Any promoter (i.e., whether isolated from nature or produced by recombinant DNA or synthetic techniques) can be used in connection with the invention to provide for transcription of the nucleic acid sequence. The promoter preferably is capable of directing transcription in a eukaryotic (desirably mammalian) cell. The functioning of the promoter can be altered by the presence of one or more enhancers and/or silencers present on the vector. "Enhancers" are cis-acting elements of DNA that stimulate or inhibit transcription

of adjacent genes. An enhancer that inhibits transcription also is termed a "silencer." Enhancers differ from DNA-binding sites for sequence-specific DNA binding proteins found only in the promoter (which also are termed "promoter elements") in that enhancers can function in either orientation, and over distances of up to several kilobase pairs (kb), even from a position downstream of a transcribed region.

[0055] Promoter regions can vary in length and sequence and can further encompass one or more DNA binding sites for sequence-specific DNA binding proteins and/or an enhancer or silencer. Enhancers and/or silencers can similarly be present on a nucleic acid sequence outside of the promoter *per se*. Desirably, a cellular or viral enhancer, such as the cytomegalovirus (CMV) immediate-early enhancer, is positioned in the proximity of the promoter to enhance promoter activity. In addition, splice acceptor and donor sites can be present on a nucleic acid sequence to enhance transcription.

[0056] Any suitable promoter or enhancer sequence can be used in the context of the invention. In this respect, the antigen-encoding nucleic acid sequence can be operably linked to a viral promoter. Suitable viral promoters include, for instance, cytomegalovirus (CMV) promoters, such as the CMV immediate-early promoter (described in, for example, U.S. Patents 5,168,062 and 5,385,839), promoters derived from human immunodeficiency virus (HIV), such as the HIV long terminal repeat promoter, Rous sarcoma virus (RSV) promoters, such as the RSV long terminal repeat, mouse mammary tumor virus (MMTV) promoters, HSV promoters, such as the Lap2 promoter or the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci., 78,* 144-145 (1981)), promoters derived from SV40 or Epstein Barr virus, an adeno-associated viral promoter, such as the p5 promoter, and the like.

[0057] Alternatively, the invention employs a cellular promoter, i.e., a promoter that drives expression of a cellular protein. Preferred cellular promoters for use in the invention will depend on the desired expression profile to produce the antigen(s). In one aspect, the cellular promoter is preferably a constitutive promoter that works in a variety of cell types, such as immune cells described herein. Suitable constitutive promoters can drive expression of genes encoding transcription factors, housekeeping genes, or structural genes common to eukaryotic cells. For example, the Ying Yang 1 (YY1) transcription factor (also referred to as NMP-1, NF-E1, and UCRBP) is a ubiquitous nuclear transcription factor that is an intrinsic component of the nuclear matrix (Guo et al., *PNAS, 92*, 10526-10530 (1995)). While the promoters described herein are considered as constitutive promoters, it is understood in the art that constitutive promoters can be upregulated. Promoter analysis shows that the elements critical for basal transcription reside from –277 to +475 of the YY1 gene relative to the transcription start site from the promoter, and include a TATA and CCAAT box. JEM-1 (also known as HGMW and BLZF-1) also is a

ubiquitous nuclear transcription factor identified in normal and tumorous tissues (Tong et al., *Leukemia*, *12*(11), 1733-1740 (1998), and Tong et al., *Genomics*, *69*(3), 380-390 (2000)). JEM-1 is involved in cellular growth control and maturation, and can be upregulated by retinoic acids. Sequences responsible for maximal activity of the JEM-1 promoter has been located at –432 to +101 of the JEM-1 gene relative the transcription start site of the promoter. Unlike the YY1 promoter, the JEM-1 promoter does not comprise a TATA box. The ubiquitin promoter, specifically UbC, is a strong constitutively active promoter functional in several species. The UbC promoter is further characterized in Marinovic et al., *J. Biol. Chem.*, *277*(19), 16673-16681 (2002).

[0058] Many of the above-described promoters are constitutive promoters. Instead of being a constitutive promoter, the promoter can be a regulatable promoter, i.e., a promoter that is up- and/or down-regulated in response to appropriate signals. The use of a regulatable promoter or expression control sequence is particularly applicable to DNA vaccine development as antigenic proteins, including viral and parasite antigens, frequently are toxic to complementing cell lines. In one embodiment, the regulatory sequences operably linked to the antigen-encoding nucleic acid sequence include components of the tetracycline expression system, e.g., tet operator sites. For instance, the antigen-encoding nucleic acid sequence is operably linked to a promoter which is operably linked to one or more tet operator sites. An adenoviral vector comprising such an expression cassette can be propagated in a complementing cell line, such as 293-ORF6 described in, for example, U.S. Patent 5,994,106 and International Patent Application Publication WO 95/34671, which comprises a nucleic acid sequence encoding a tet repressor protein. By producing the tet repressor protein in the complementing cell line, antigen production is inhibited and propagation proceeds without any associated antigen-mediated toxicity. Suitable regulatable promoter systems also include, but are not limited to, the IL-8 promoter, the metallothionine inducible promoter system, the bacterial lacZYA expression system, and the T7 polymerase system. Further, promoters that are selectively activated at different developmental stages (e.g., globin genes are differentially transcribed from globin-associated promoters in embryos and adults) can be employed. The promoter sequence can contain at least one regulatory sequence responsive to regulation by an exogenous agent. The regulatory sequences are preferably responsive to exogenous agents such as, but not limited to, drugs, hormones, radiation, or other gene products.

[0059] The promoter can be a tissue-specific promoter, i.e., a promoter that is preferentially activated in a given tissue and results in expression of a gene product in the tissue where activated. A tissue-specific promoter suitable for use in the invention can be chosen by the ordinarily skilled artisan based upon the target tissue or cell-type. Preferred tissue-specific promoters for use in the inventive method are specific to immune cells, such

as the dendritic-cell specific Dectin-2 promoter described in Morita et al., *Gene Ther.*, *8*, 1729-37 (2001).

[0060] In yet another embodiment, the promoter can be a chimeric promoter. A promoter is "chimeric" in that it comprises at least two nucleic acid sequence portions obtained from, derived from, or based upon at least two different sources (e.g., two different regions of an organism's genome, two different organisms, or an organism combined with a synthetic sequence). Preferably, the two different nucleic acid sequence portions exhibit less than about 40%, more preferably less than about 25%, and even more preferably less than about 10% nucleic acid sequence identity to one another (which can be determined by methods described elsewhere herein). Any suitable chimeric promoter can be used in the inventive method. Preferably, the chimeric promoter is comprised of a functional portion of a viral promoter and a functional portion of a cellular promoter. More preferably, the chimeric promoter comprises a functional portion of a viral promoter and a functional portion of a cellular promoter that is radiation-inducible. Most preferably, the chimeric promoter comprises a functional portion of a CMV promoter and a functional portion of an EGR-1 promoter (i.e., a chimeric "CMV/EGR-1" promoter). The functional portion of the CMV promoter preferably is derived from a human CMV, and more particularly from the human CMV immediate early (IE) promoter/enhancer region (see, e.g., U.S. Patents 5,168,062 and 5,385,839). In addition, the functional portion of the EGR-1 promoter preferably comprises one or more CArG domains of an EGR-1 promoter, as described in, for example, U.S. Patents 6,579,522 and 6,605,712. In a particularly preferred embodiment of the invention, the chimeric promoter comprises a functional portion of the CMV IE enhancer/promoter region, and an EGR-1 promoter comprising six CArG domains. In this manner, the portion of the CMV IE enhancer/promoter region functions as an enhancer for the EGR-1 promoter. Chimeric promoters can be generated using standard molecular biology techniques, such as those described in Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994).

[0061] In accordance with the invention, a "functional portion" is any portion of a promoter that measurably promotes, enhances, or controls expression (typically transcription) of an operatively linked nucleic acid. Such regulation of expression can be measured via RNA or protein detection by any suitable technique, and several such techniques are known in the art. Examples of such techniques include Northern analysis (see, e.g., Sambrook et al., *supra*, and McMaster and Carmichael, *PNAS, 74*, 4835-4838 (1977)), RT-PCR (see, e.g., U.S. Patent 5,601,820, and Zaheer et al., *Neurochem Res.*, *20*, 1457-1463 (1995)), *in situ* hybridization methods (see, e.g., U.S. Patents 5,750,340 and

5,506,098), antibody-mediated techniques (see, e.g., U.S. Patents 4,367,110, 4,452,901, and 6,054,467), and promoter assays utilizing reporter gene systems such as the luciferase gene (see, e.g., Taira et al., *Gene, 263*, 285-292 (2001)). Eukaryotic expression systems in general are further described in Sambrook et al., *supra.*

[0062] A promoter can be selected for use in the method of the invention by matching its particular pattern of activity with the desired pattern and level of expression of the antigen(s). For example, the adenoviral vector can comprise two or more nucleic acid sequences that encode different antigens and are operably linked to different promoters displaying distinct expression profiles. For example, a first promoter is selected to mediate an initial peak of antigen production, thereby priming the immune system against an encoded antigen. A second promoter is 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 chimeric promoter can be constructed which combines the desirable aspects of multiple promoters. For example, a CMV-RSV hybrid promoter combining the CMV promoter's initial rush of activity with the RSV promoter's high maintenance level of activity is especially preferred for use in many embodiments of the inventive method. In that 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.

[0063] To optimize protein production, preferably the nucleic acid sequence encoding the antigen further comprises a polyadenylation site following the coding sequence of the antigen-encoding nucleic acid sequence. Any suitable polyadenylation sequence can be used, including a synthetic optimized sequence, as well as 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 (Human Sarcoma Virus-40) polyadenylation sequence. Also, preferably all the proper transcription signals (and translation signals, where appropriate) are correctly arranged such that the nucleic acid sequence is properly expressed in the cells into which it is introduced. If desired, the nucleic acid sequence also can incorporate splice sites (i.e., splice acceptor and splice donor sites) to facilitate mRNA production.

[0064] If the antigen-encoding nucleic acid sequence encodes a processed or secreted protein or peptide, or a protein that acts intracellularly, preferably the antigen-encoding nucleic acid sequence further comprises the appropriate sequences for processing, secretion, intracellular localization, and the like. The antigen-encoding 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, *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 02/48377.

[0065] The antigen protein encoded by the nucleic acid sequence of the adenoviral vector also can be modified to attach or incorporate the antigen on the host cell surface. In this respect, the antigen can comprise a membrane anchor, such as a gpi-anchor, for conjugation onto the cell surface. A transmembrane domain can be fused to the antigen to incorporate a terminus of the antigen protein into the cell membrane. Other strategies for displaying peptides on a cell surface are known in the art and are appropriate for use in the context of the invention.

[0066] In the method of the invention, the adenoviral vector preferably is administered to a mammal (e.g., a human), wherein the nucleic acid sequence encoding the antigen is expressed to induce an immune response against the antigen. 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 infectious agent comprising the antigen. However, protective immunity is not required in the context of the invention. The inventive method further can be used for antibody production and harvesting.

[0067] To enhance the immune response generated against the antigen, the adenoviral vector, or a different gene transfer vector administered to the mammal, can comprise a nucleic acid sequence that encodes an immune stimulator, such as a cytokine, a chemokine, or a chaperone. Cytokines include, for example, Macrophage Colony Stimulating Factor (e.g., GM-CSF), Interferon Alpha (IFN-α), 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 (IL8), 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 (MCP-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 Hsp170, Hsc70, and Hsp40. Cytokines and chemokines are generally described in the art, including the Invivogen catalog (2002), San Diego, CA.

[0068] The invention can comprise administering multiple adenoviral vectors to the mammal, each adenoviral vector comprising one or more nucleic acid sequences encoding one or more antigens and/or immunomodulators. If the adenoviral vector comprises more than one antigen-encoding nucleic acid sequence, two or more nucleic acid sequences can be operably linked to the same promoter (e.g., to form a bicistronic sequence), two or more nucleic acid sequences can be operably linked to separate promoters of the same type (e.g., the CMV promoter), or two or more nucleic acid sequences can be operably linked to separate and different promoters (e.g., the CMV promoter and β-actin promoter). The multiple adenoviral vectors can include two or more adenoviral vector constructs encoding different antigens, different epitopes of the same antigenic protein, the same antigenic protein derived from different species or clades of microorganism, antigens from different microorganisms, and the like. In will be appreciated that, in some embodiments, administering a "cocktail" of adenoviral vectors encoding different antigens or different epitopes of the same antigen can provide a more effective immune response than administering a single adenoviral vector clone to a mammal.

[0069] Likewise, administering the adenoviral vector encoding an antigen can be one component of a multistep regimen for inducing an immune response in a mammal. In particular, the inventive method can represent one arm of a prime and boost immunization regimen. The inventive method, therefore, can comprise administering to the mammal a priming gene transfer vector comprising a nucleic acid sequence encoding at least one antigen prior to administering the adenoviral vector. The antigen encoded by the priming gene transfer vector can be the same or different from the antigen of the adenoviral vector. The adenoviral vector is then administered to boost the immune response to a given pathogen. More than one boosting composition comprising the adenoviral vector can be provided in any suitable timeframe (e.g., at least about 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks, 16 weeks, or more following priming) to maintain immunity.

[0070] Any gene transfer vector can be employed as a priming gene transfer vector, including, but not limited to, a plasmid, a retrovirus, an adeno-associated virus, a vaccinia virus, a herpesvirus, or an adenovirus. Ideally, the priming gene transfer vector is a plasmid or an adenoviral vector. Alternatively, an immune response can be primed or boosted by administration of the antigen itself, e.g., an antigenic protein, inactivated pathogen, and the like.

[0071] Any route of administration can be used to deliver the adenoviral vector to the mammal. Indeed, although more than one route can be used to administer the adenoviral

vector, a particular route can provide a more immediate and more effective reaction than another route. Preferably, the adenoviral vector is administered via intramuscular injection. A dose of adenoviral vector 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.

[0072] 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), 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.

[0073] The dose of adenoviral vector administered to the mammal will depend on a number of factors, including the size of a target tissue, the extent of any side-effects, the particular route of administration, and the like. The dose ideally comprises an "effective amount" of adenoviral vector, i.e., a dose of adenoviral vector which provokes a desired immune response in the mammal. The desired immune response can entail production of antibodies, protection upon subsequent challenge, immune tolerance, immune cell activation, and the like. Desirably, a single dose of adenoviral vector comprises at least about $1x10^5$ particles (which also is referred to as particle units) of the adenoviral vector. The dose preferably is at least about $1x10^6$ particles (e.g., about $1x10^6$-$1x10^{12}$ particles), more preferably at least about $1x10^7$ particles, more preferably at least about $1x10^8$ particles (e.g., about $1x10^8$-$1x10^{11}$ particles), and most preferably at least about $1x10^9$ particles (e.g., about $1x10^9$-$1x10^{10}$ particles) of the adenoviral vector. Alternatively, the dose comprises no more than about $1x10^{14}$ particles, preferably no more than about $1x10^{13}$ particles, even more preferably no more than about $1x10^{12}$ particles, even more preferably no more than about $1x10^{11}$ particles, and most preferably no more than about $1x10^{10}$ particles (e.g., no more than about $1x10^9$ particles). In other words, a single dose of adenoviral vector can comprise, for example, about $1x10^6$ particle units (pu), $2x10^6$ pu, $4x10^6$ pu, $1x10^7$ pu, $2x10^7$ pu, $4x10^7$ pu, $1x10^8$ pu, $2x10^8$ pu, $4x10^8$ pu, $1x10^9$ pu, $2x10^9$ pu, $4x10^9$ pu, $1x10^{10}$ pu, $2x10^{10}$ pu, $4x10^{10}$ pu, $1x10^{11}$ pu, $2x10^{11}$ pu, $4x10^{11}$ pu, $1x10^{12}$ pu, $2x10^{12}$ pu, or $4x10^{12}$ pu of the adenoviral vector.

[0074] The adenoviral vector desirably is administered in a composition, preferably a physiologically acceptable (e.g., pharmaceutically acceptable) composition, which

comprises a carrier, preferably a physiologically (e.g., pharmaceutically) acceptable carrier and the adenoviral vector(s). 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 composition preferably is free of replication-competent adenovirus. The composition can optionally be sterile or sterile with the exception of the inventive adenoviral vector.

[0075] Suitable formulations for the composition include aqueous and non-aqueous solutions, 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 of the kind previously described. Preferably, the carrier is a buffered saline solution. More preferably, the adenoviral vector for use in the inventive method is administered in a composition formulated to protect the expression 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 expression vector, such as glassware, syringes, or needles. The composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the expression 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 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, 6,514,943, U.S. Patent Application Publication No. 2003/0153065 A1, and International Patent Application Publication WO 00/34444. A 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 viral vector. As discussed herein, immune system stimulators can be administered to enhance any immune response to the antigen.

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.

[0076] The construction of adenoviral vectors is well understood in the art. Adenoviral vectors can be constructed and/or purified using the methods set forth, for example, in U.S. Patents 5,965,358, 6,168,941, 6,329,200, 6,383,795, 6,440,728, 6,447,995, 6,475,757, 6,573,092, and 6,586,226, and U.S. Patent Application Publication Nos. 2003/0170899 A1, 2003/0203469 A1, and 2003/0203480 A1, and International Patent Application Publications WO 98/53087, WO 98/56937, WO 99/15686, WO 99/54441, WO 00/12765, WO 01/77304, WO 02/29388, WO 02/31169, and WO 03/39459 as well as the other references identified herein. Non-group C adenoviral vectors, including adenoviral serotype 35 vectors, can be produced using the methods set forth in, for example, U.S. Patents 5,837,511 and 5,849,561, and International Patent Application Publications WO 97/12986 and WO 98/53087. Moreover, numerous adenoviral vectors are available commercially.

[0077] Replication-deficient adenoviral vectors are typically produced in complementing cell lines that provide gene functions not present in the replication-deficient adenoviral vectors, but required for viral propagation, at appropriate levels in order to generate high titers of viral vector stock. Desirably, the complementing cell line comprises, integrated into the cellular genome, adenoviral nucleic acid sequences which encode gene functions required for adenoviral propagation. A preferred cell line complements for at least one and preferably all replication-essential gene functions not present in a replication-deficient adenovirus. The complementing cell line can complement for a deficiency in at least one replication-essential gene function encoded by the early regions, late regions, viral packaging regions, virus-associated RNA regions, or combinations thereof, including all adenoviral functions (e.g., to enable propagation of adenoviral amplicons). Most preferably, the complementing cell line complements for a deficiency in at least one replication-essential gene function (e.g., two or more replication-essential gene functions) of the E1 region of the adenoviral genome, particularly a deficiency in a replication-essential gene function of each of the E1A and E1B regions. In addition, the complementing cell line can complement for a deficiency in at least one replication-essential gene function of the E2 (particularly as concerns the adenoviral DNA polymerase and terminal protein) and/or E4 regions of the adenoviral genome. Desirably, a cell that complements for a deficiency in the E4 region comprises the E4-ORF6 gene sequence and produces the E4-ORF6 protein. Such a cell desirably comprises at least ORF6 and no other ORF of the E4 region of the adenoviral genome. The cell line preferably is further characterized in that it contains the complementing genes in a non-overlapping fashion with the adenoviral vector, which minimizes, and practically eliminates, the possibility of the vector genome recombining

with the cellular DNA. Accordingly, the presence of replication competent adenoviruses (RCA) is minimized if not avoided in the vector stock, which, therefore, is suitable for certain therapeutic purposes, especially vaccination purposes. The lack of RCA in the vector stock avoids the replication of the adenoviral vector in non-complementing cells. Construction of such a complementing cell lines involve standard molecular biology and cell culture techniques, such as those described by Sambrook et al., *supra,* and Ausubel et al., *supra*).

[0078] Complementing cell lines for producing the adenoviral vector 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 97/00326, and U.S. Patents 5,994,128 and 6,033,908), and 293-ORF6 cells (described in, e.g., International Patent Application Publication WO 95/34671 and Brough et al., *J. Virol.*, *71*, 9206-9213 (1997)). Additional complementing cells are described in, for example, U.S. Patents 6,677,156 and 6,682,929, and International Patent Application Publication WO 03/20879. 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 desired adenoviral vector. Helper virus is often engineered to prevent packaging of infectious helper virus. For example, one or more replication-essential gene functions of the E1 region of the adenoviral genome are provided by the complementing cell, while one or more replication-essential gene functions of the E4 region of the adenoviral genome are provided by a helper virus.

[0079] This following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0080] This example demonstrates a method of inducing an immune response in a mammal comprising administering to the mammal an adenoviral vector comprising (a) a subgroup C fiber protein wherein a native coxsackievirus and adenovirus receptor (CAR)-binding site is disrupted, (b) a subgroup C penton base protein wherein a native integrin-binding site is disrupted, and (c) a nucleic acid sequence encoding an antigen.

[0081] Adenoviral serotype 5 E1/E3/E4-deficient adenoviral vectors containing, in place of the deleted E1 region, a nucleic acid sequence encoding the green fluorescent protein (GFP) operably linked to the CMV promoter were generated. To reduce adenoviral fiber-mediated transduction via CAR, the CAR-binding domain of the adenoviral fiber

protein and the integrin-binding domain of the adenoviral penton base protein were disrupted (Adf.DA-HA) ("double ablation" vector). For comparison, a corresponding GFP-expressing adenoviral vector containing wild type capsid proteins (Adf.11D) also was generated. Adtgp140 is an E1/E3/E4-deficient serotype 5 adenoviral vector that does not express GFP, and served as a negative control.

[0082] Each of the above-described adenoviral vectors was injected into the hind leg muscles of mice at a dose of 1 x 10^9 particle units (pu). Spleen cells were analyzed for reactivity against a GFP antigen by contacting spleen cells with a GFP peptide pool at two weeks post injection. The percentage of immune cells (i.e., $CD4^+$ and $CD8^+$ T lymphocytes) reactive to the GFP antigen was determined using an intracellular flow analysis, as described in, for example, Yang et al., *J Virol.*, *77*(1), 799-803 (2003).

[0083] The results of this analysis are shown in Figures 1A and 1B. The results demonstrate that the percentage of GFP-reactive immune cells elicited by the double-ablation vector (Adf.DA-HA) was substantially the same as the percentage of GFP-reactive immune cells elicited by the wild-type capsid vector (Adf.11D).

[0084] This example demonstrates the ability of a subgroup C adenoviral vector ablated for native binding, i.e., by disruption of the CAR-binding and integrin-binding domains of the adenovirus fiber and penton base proteins, respectively, to efficiently induce an immune response against an antigen in a mammal.

EXAMPLE 2

[0085] This example demonstrate the ability of a subgroup C adenoviral vector ablated for native binding to efficiently transduce professional antigen presenting cells.

[0086] A double ablation adenoviral vector encoding the luciferase gene instead of GFP (Adf.DA-HA.luc) was generated as described in Example 1. The specificity of Adf.DA-HA.luc was evaluated in murine bone marrow-derived dendritic cells (DC). Specifically, murine bone marrow (BM) dendritic cells were infected with Adf.DA-HA.luc in cells gated for the CD19 and CD11c dendritic cell markers. For comparison, corresponding GFP-expressing and luciferase-expression adenoviral vectors containing wild type capsid proteins also were tested. A dose-response analysis was performed with different multiplicity of infections (MOI) in BM or plasmacytoid dendritic cells of mouse or human origin.

[0087] Adf.DA-HA.luc readily infected bone marrow cells (see Figures 2A and 2B), and Cd19-Cd11c+ cells (see Figure 2A). Adf.DA-HA.luc also transduced human dendritic cell types, including plasmacytoid dendritic cells. While Adf.DA-HA.luc showed slightly lower transduction efficiencies, as measured by slightly reduced luciferase reporter activity

per input viral particle in these cells, the vector showed comparable activity over a two-log range of multiplicities of infection (see Figures 2B and 2C).

[0088] The results of this example demonstrate that a doubly ablated subgroup C adenoviral vector can transduce antigen presenting cells independently of fiber-CAR and penton base-integrin interactions.

[0089] 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.

[0090] 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.

[0091] 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.

WHAT IS CLAIMED IS:

1. A method of inducing an immune response in a mammal, which method comprises administering to the mammal an adenoviral vector comprising (a) a subgroup C fiber protein wherein a native coxsackievirus and adenovirus receptor (CAR)-binding site is disrupted, (b) a subgroup C penton base protein wherein a native integrin-binding site is disrupted, and (c) a nucleic acid sequence encoding at least one antigen which is expressed in the mammal to induce an immune response, wherein the antigen is derived from an infectious agent other than an adenovirus.

2. The method of claim 1, wherein the adenoviral vector is a serotype 2 adenoviral vector.

3. The method of claim 1, wherein the adenoviral vector is a serotype 5 adenoviral vector.

4. The method of any of claims 1-3, wherein the adenoviral vector is replication-competent.

5. The method of any of claims 1-3, wherein the adenoviral vector is conditionally-replicating.

6. The method of any of claims 1-3, wherein the adenoviral vector is replication-deficient.

7. The method of claim 6, wherein the adenoviral vector comprises an adenoviral genome that is deficient in one or more replication-essential gene functions of the E1 region of the adenoviral genome.

8. The method of claim 7, wherein the adenoviral vector comprises an adenoviral genome that is deficient in all replication-essential gene functions of the E1A and E1B regions of the adenoviral genome.

9. The method of any of claims 1-8, wherein the adenoviral vector comprises an adenoviral genome that is deficient in one or more gene functions of the E3 region of the adenoviral genome.

10. The method of any of claims 6-9, wherein the adenoviral vector comprises an adenoviral genome that is deficient in one or more replication-essential gene functions of the E4 region of the adenoviral genome.

11. The method of claim 10, wherein a spacer sequence is positioned in the E4 region of the adenoviral genome.

12. The method of claim 10, wherein the nucleic acid sequence encoding the antigen is positioned in the E4 region of the adenoviral genome.

13. The method of any of claims 7-11, wherein the nucleic acid sequence encoding the antigen is positioned in the E1 region of the adenoviral genome.

14. The method of any of claims 1-13, wherein the penton base protein lacks a native RGD sequence.

15. The method of any of claims 1-13, wherein the penton base protein comprises a native RGD sequence that is conformationally inaccessible for binding to the α_v integrin receptor.

16. The method of any of claims 1-15, wherein the fiber protein lacks the fiber knob.

17. The method of any of claims 1-16, wherein the fiber protein trimerizes.

18. The method of any of claims 1-17, wherein the adenoviral vector comprises a chimeric adenoviral coat protein comprising a non-native amino acid sequence that binds a cellular receptor.

19. The method of claim 18, wherein the non-native amino acid sequence comprises an RGD motif.

20. The method of any of claims 1-19, wherein the adenoviral vector comprises multiple nucleic acid sequences encoding different antigens.

21. The method of claim 20, wherein two or more nucleic acid sequences encoding different antigens are operably linked to different promoters.

22. The method of any of claims 1-19, wherein the adenoviral vector comprises multiple nucleic acid sequences encoding the same antigen.

23. The method of claim 22, wherein two or more nucleic acid sequences encoding the same antigen are operably linked to different promoters.

24. The method of any of claims 1-23, wherein the infectious agent is a virus.

25. The method of claim 24, wherein at least one antigen is selected from the group consisting of env, gag, and pol from clades A, B, or C of a human immunodeficiency virus (HIV), and a fusion protein comprising any of the foregoing.

26. The method of claim 24, wherein at least one antigen is selected from the group consisting of an E protein, an M protein, and a spike protein of a severe acute respiratory syndrome (SARS) virus.

27. The method of any of claims 1-26, wherein the adenoviral vector comprises a nucleic acid sequence encoding an immune stimulator.

28. The method of any of claims 1-27, wherein the mammal is a human.

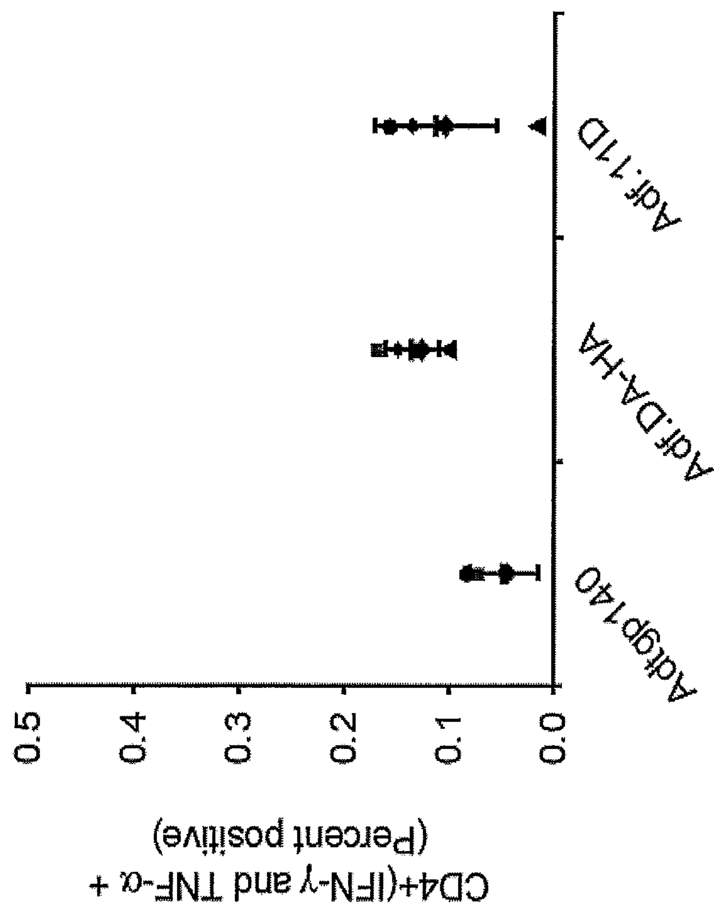


Figure 1A

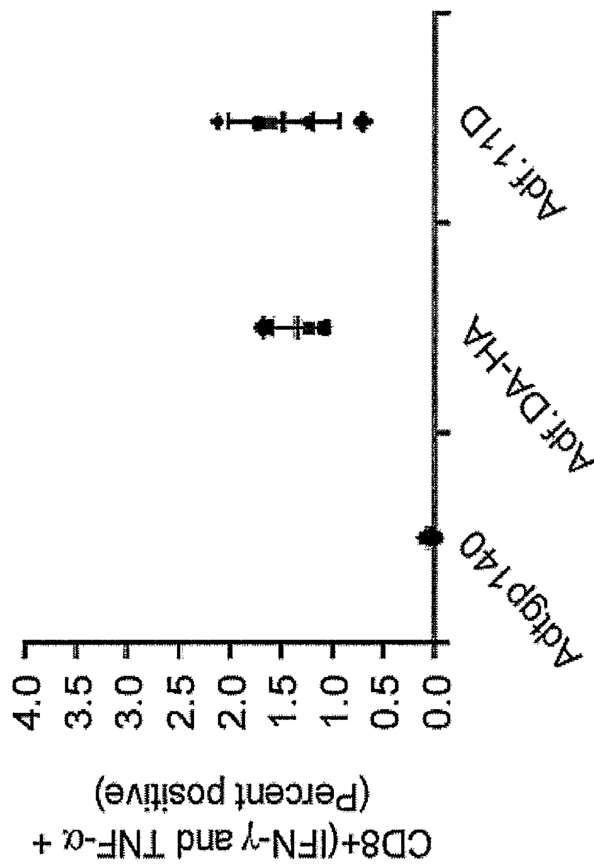


Figure 1B

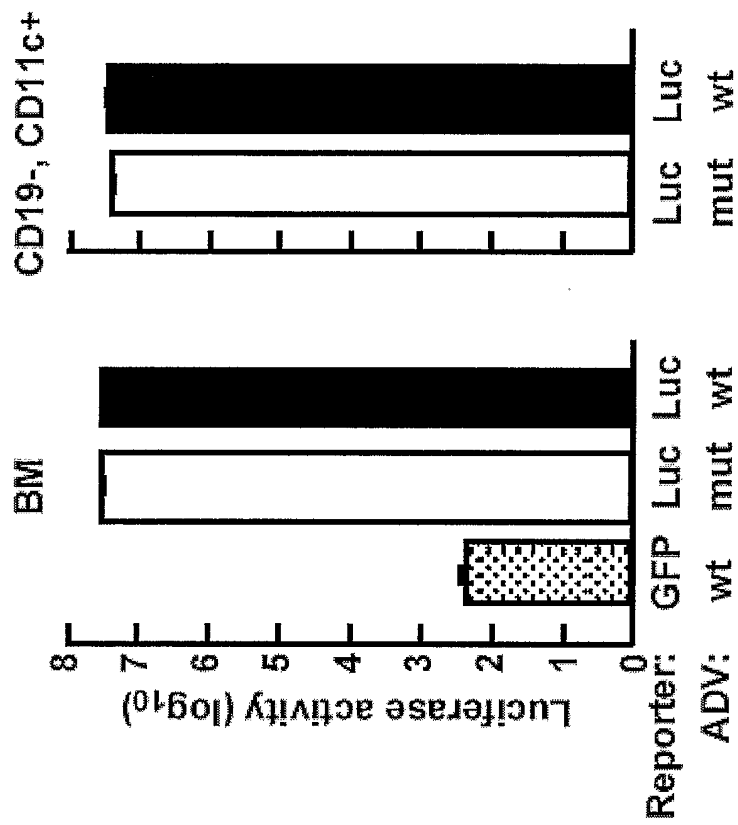


Figure 2A

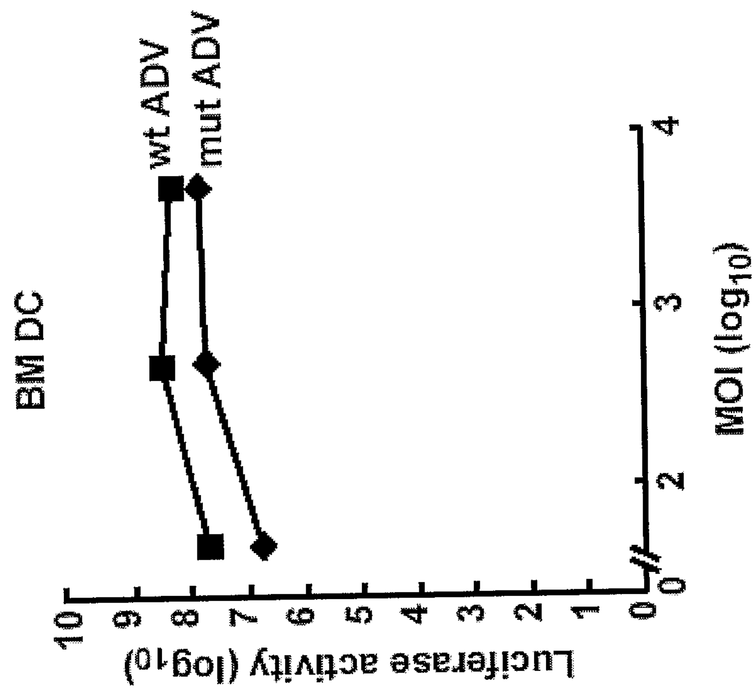


Figure 2B

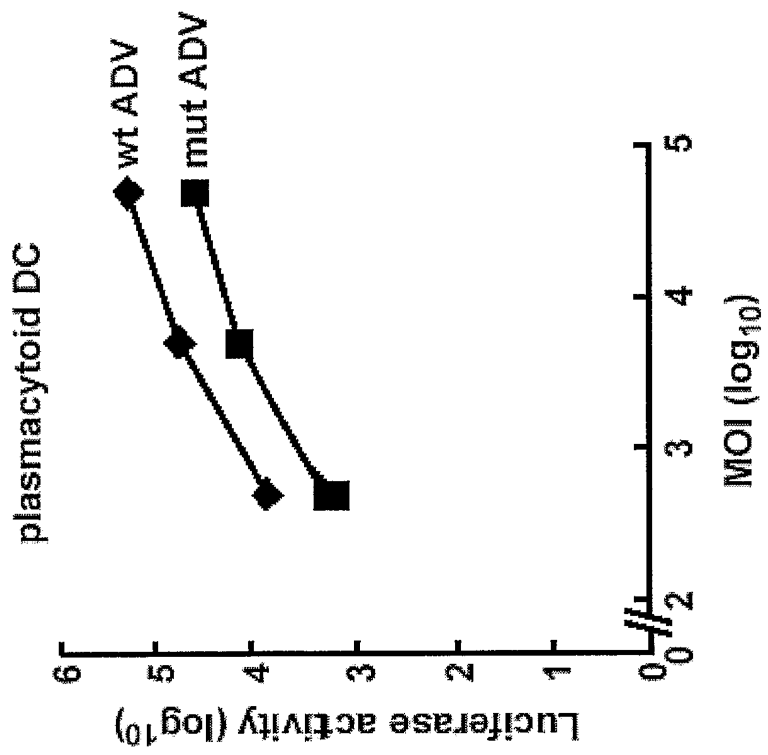


Figure 2C

238438.ST25
SEQUENCE LISTING

<110> THE GOVERNMENT OF THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES
GENVEC, INC.
NABEL, Gary J.
CHENG, Cheng
GALL, Jason G. D.
WICKHAM, Thomas J.

<120> METHOD OF USING ADENOVIRAL VECTORS WITH INCREASED IMMUNOGENECITY IN VIVO

<130> 238438-GEN

<150> US 60/606,273
<151> 2004-08-31

<160> 3

<170> PatentIn version 3.3

<210> 1
<211> 5
<212> PRT
<213> Artificial

<220>
<223> Synthetic peptide

<400> 1

Cys Arg Gly Asp Cys
1   5

<210> 2
<211> 9
<212> PRT
<213> Artificial

<220>
<223> Synthetic peptide

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> "Xaa" may be any amino acid.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> "Xaa" may be any amino acid.

<400> 2

Cys Xaa Cys Arg Gly Asp Cys Xaa Cys
1   5

<210> 3
<211> 9
<212> PRT
<213> Artificial

<220>
<223> Synthetic peptide

<400> 3

```
Cys Asp Cys Arg Gly Asp Cys Phe Cys
1               5
```