

DNA VACCINE FOR HUMAN PAPILLOMAVIRUS AND METHOD FOR USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 63/117,473, filed November 24, 2020, and entitled “HUMAN PAPILLOMAVIRUS DNA VACCINE,” which is hereby fully incorporated by reference herein.

REFERENCE TO SEQUENCE LISTING

[0002] Accompanying this application is a sequence listing in an American Standard Code for Information Interchange (ASCII) text file named “211101-sequence-listing-v1F-txt”, created November 23, 2021, and having a size of 19,922 bytes. The sequence listing is hereby fully incorporated by reference herein.

BACKGROUND

1. Field

[0003] The present disclosure generally relates to DNA vaccines, particularly for an HPV-associated disease, and more specifically to improving the DNA vaccine through codon optimization.

2. Related Art

[0004] Human papillomavirus (HPV) is a common etiological agent in several human cancers, including cervical, anal, penile, vulvar, vaginal, and head and neck cancers. Current

vaccines against HPV, such as Gardasil[®] and Cervarix[®], have shown clinical efficacy in preventing HPV infection, but they are ineffective in treating patients with existing HPV infection or HPV-associated cancers. As such, development of therapeutic vaccines for patients infected with HPV or even suffering from HPV-associated diseases are highly demanded.

[0005] Deoxyribonucleic acid (DNA) vaccination is a technique for protecting against infection or treating disease by injection with a genetically engineered plasmid containing a DNA sequence encoding one or more antigens. DNA vaccines have theoretical advantages over conventional vaccines, including safety, speed, and predictability of manufacture; temperature stability; flexibility in design; and the ability to induce a wider range of immune response types. Despite the advantages described above, DNA vaccines generally have obstacles to induce strong antigen-specific immune responses in animals. For example, DNA vaccination with HPV E6 and E7 genes may be poorly immunogenic because the virus has evolved mechanisms to evade host recognition, including low levels of E6/E7 expression. Therefore, a large number of different strategies to enhance DNA vaccine immunogenicity have been tested, including vector design improvement, antigen codon optimization, use of traditional adjuvant and molecular adjuvants, electroporation (EP), co-expression of molecular adjuvants and prime-boost strategies (L. Li and N. Petrovsky, *Expert Rev Vaccines*. 2016;15(3):313-29).

[0006] Codon optimization refers to approaches using synonymous mutations to increase protein expression of an interested gene, such as the antigen-expressing gene in the DNA vaccine. However, codon optimization does not always positively correlate with DNA vaccine efficacy. Many studies have shown that rare codons may not always be a speed-limiting step and frequently used codons do not guarantee increased protein production. Moreover, coding regions not only specify amino acid sequences, but often also contain overlapping genetic information which includes RNA secondary structures that can affect protein folding. It is indicated that synonymous codons could potentially change protein conformation and function (L. Li and N. Petrovsky, *Expert Rev Vaccines*. 2016;15(3):313-29, V. P. Mauro and S. A. Chappell, *Methods*

Mol Biol. 2018;1850:275-288). Accordingly, using codon optimization to enhance expression of encoded antigens in DNA vaccines may cause unexpected detrimental results and requires careful consideration. Further, it is even more difficult to predict whether a DNA vaccine comprising codon-optimized sequences can achieve a desired immunogenicity.

[0007] In view of the reasons mentioned above, there exists an unmet need for providing a DNA vaccine with better efficacy in treating HPV-associated diseases. However, improving the efficacy of DNA vaccines against HPV-associated diseases by way of codon optimization is still highly unpredictable and thus remains a problem to be solved in the field.

BRIEF SUMMARY OF THE DISCLOSURE

[0008] In some embodiments of the present disclosure, a DNA vaccine for a subject having a human papillomavirus (HPV)-associated disease may be provided. The DNA vaccine may include a DNA construct including a fusion gene. The fusion gene may be a subsegment of the DNA construct that includes an optimized HPV subsequence encoding at least one HPV antigen. The optimized HPV subsequence may include one or more of: an HPV-16 E6 expressing gene set forth in SEQ ID NO: 1, an HPV-16 E7 expressing gene set forth in SEQ ID NO: 2, an HPV-18 E6 expressing gene set forth in SEQ ID NO: 3, and an HPV-18 E7 expressing gene set forth in SEQ ID NO: 4.

[0009] In some embodiments, the fusion gene may further include a subsequence encoding an immunostimulant.

[0010] In some embodiments, the immunostimulant may be a 70 kilodalton (kDa) heat shock protein (HSP70) or a human calreticulin (CRT) protein.

[0011] In some embodiments, the optimized HPV subsequence may include the HPV-16 E6, HPV-16 E7, HPV-18 E6, and HPV-18 E7 expressing gene, and the fusion gene may include SEQ ID NO: 5.

[0012] In some embodiments, the fusion gene may further include an optimized signal

sequence, and the fusion gene may include SEQ ID NO: 6.

[0013] Another aspect of present disclosure may provide a method for treating an HPV-associated disease in a subject in need thereof. The method may include administering the aforementioned DNA vaccine to the subject.

[0014] In some embodiments, the method may further include administering a recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 to the subject, where the DNA vaccine is administered as a priming vaccine and the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 is administered as a boosting vaccine.

[0015] In some embodiments, the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 may be TA-HPV.

[0016] In some embodiments, the TA-HPV may be administered at a dose ranging from 1×10^4 plaque-forming units (pfu) to 2×10^8 pfu.

[0017] In some embodiments, the DNA vaccine may be administered at a dose ranging from 100 micrograms per subject to 20 milligrams per subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] So that the manner in which the above-recited features of the present disclosure can be understood in detail, a more particular description of the disclosure, briefly summarized above, may be had by reference to embodiments, some of which are illustrated in the appended drawings. It is to be noted, however, that the appended drawings illustrate only typical embodiments of this disclosure and are therefore not to be considered limiting of its scope, for the disclosure may admit to other equally effective embodiments.

[0019] FIG. 1 is a schematic illustration showing DNA constructs according to some embodiments of the present disclosure.

[0020] FIG. 2 is a western blot image showing the HPV antigen expression level in Human Embryonic Kidney (HEK) 293 cells transfected with either pBI-1, pBI-10.1, pBI-11, or pBI-12,

in accordance with an embodiment of the present disclosure.

[0021] FIGS. 3A and 3B are bar charts comparing the percentage of IFN- γ ⁺ CD8⁺ T cells/total CD 8⁺ cells of a control group, a pBI-10.1 group, a pBI-11 group and a pBI-12 group in the *in vitro* T cell activation assays using either murine H-2D^b-restricted HPV-16 E7 peptide (aa 49-57)-specific CD8⁺ T cells (FIG. 3A) or H-2K^b-restricted HPV-18 E6 peptide (aa 67-75)-specific CD8⁺ T cells (FIG. 3B) to compare the capacity of HPV antigen presentation by cells transfected with each of the DNA constructs, in accordance with an embodiment of the present disclosure.

[0022] FIG. 4A is a schematic illustration of the experimental design for comparing HPV-16 E7-specific CD8⁺ T cell responses generated by the various DNA constructs, in accordance with an embodiment of the present disclosure.

[0023] FIG. 4B is a bar chart comparing the number of HPV-16 E7-specific IFN- γ ⁺ CD8⁺ T cells/ 3×10^5 splenocytes after the splenocytes prepared from mice vaccinated with either pBI-10.1, pBI-11, or pBI-12 being stimulated by HPV-16 E7 (aa 49-57) peptide, in accordance with an embodiment of the present disclosure.

[0024] FIG. 5A is a schematic illustration of the experimental design of an *in vivo* tumor treatment experiment using HPV-16 E6/E7⁺ TC-1 tumor model, in accordance with an embodiment of the present disclosure.

[0025] FIG. 5B is a bar chart showing the percentage of HPV-16 E7-specific CD8⁺ T cells/total CD 8⁺ cells in peripheral blood mononuclear cells (PBMCs) prepared from untreated mice or mice treated with pBI-10.1, pBI-11, or pBI-12 using HPV-16 E7 (aa 49-57) peptide-loaded tetramer staining, using a two-tailed Student's t-test, in accordance with an embodiment of the present disclosure.

[0026] FIG. 5C is a bar chart showing the percentage of HPV-18 E6-specific CD8⁺ T cells/total CD 8⁺ cells in PBMCs prepared from untreated mice or mice treated with pBI-10.1, pBI-11, or pBI-12 using HPV-18 E6 (aa 67-75) peptide-loaded tetramer staining, using a two-tailed Student's t-test, in accordance with an embodiment of the present disclosure.

[0027] FIG. 5D is a curve chart showing TC-1 tumor volumes in the untreated mice and the mice treated with pBI-10.1, pBI-11, or pBI-12 in the *in vivo* tumor treatment experiment, in accordance with an embodiment of the present disclosure.

[0028] FIG. 5E is a Kaplan-Meier survival curve showing the probability of survival in the untreated mice and the mice treated with pBI-10.1, pBI-11, or pBI-12 in the *in vivo* tumor treatment experiment, in accordance with an embodiment of the present disclosure.

[0029] In accordance with common practice, the various described features are not drawn to scale and are drawn to emphasize features relevant to the present disclosure. Like reference characters denote like elements throughout the figures and text.

DETAILED DESCRIPTION

[0030] The present disclosure will now be described more fully hereinafter with reference to the accompanying drawings, in which exemplary embodiments of the disclosure are shown. This disclosure may, however, be embodied in many different forms and should not be construed as limited to the exemplary embodiments set forth herein. Rather, these exemplary embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the disclosure to those skilled in the art. Like reference numerals refer to like elements throughout.

[0031] The terminology used herein is for the purpose of describing particular exemplary embodiments only and is not intended to be limiting of the disclosure. As used herein, the singular forms “a”, “an”, and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms “comprises” and/or “comprising,” or “includes” and/or “including” or “has” and/or “having”, when used herein, specify the presence of stated features, regions, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, regions, integers, steps, operations, elements, components, and/or groups thereof.

[0032] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the present disclosure, and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[0033] A DNA vaccine of the present disclosure is provided for a subject having a human papillomavirus (HPV)-associated disease. The subject having an HPV-associated disease may be a mammal (e.g., human, mice, etc.) suffering from, but not limited to, warts, papilloma, intraepithelial neoplasia, penile cancer, vaginal cancer, vulva cancer, anal cancer, oropharyngeal cancer, non-melanoma skin cancer, conjunctival cancer, or cervical cancer.

[0034] The DNA vaccine may include a DNA construct including a fusion gene, wherein the fusion gene includes a human papillomavirus (HPV) subsequence having been codon-optimized. The DNA vaccine may also be called an optimized DNA vaccine in the context described herein.

[0035] A synthetic fusion gene including a codon-optimized subsequence encoding at least one HPV antigen (e.g., an optimized HPV subsequence) may be provided. Specifically, the optimized HPV subsequence may include one or more of: an HPV-16 E6 expressing gene that encodes HPV-16 E6 antigen set forth in SEQ ID NO:1; an HPV-16 E7 expressing gene that encodes HPV-16 E7 antigen set forth in SEQ ID NO: 2; an HPV-18 E6 expressing gene that encodes HPV-18 E6 antigen set forth in SEQ ID NO: 3, and an HPV-18 E7 expressing gene that encodes HPV-18 E7 antigen set forth in SEQ ID NO: 4.

[0036] In some embodiments, the fusion gene may be cloned to a pNGVL4a plasmid, which is functioned as a vector, to provide a DNA construct, and the fusion gene therefore may become a subsegment of the DNA construct. In further embodiments, alternative and/or additional plasmids may be used to provide a vector described herein.

[0037] In some embodiments, the DNA vaccine may further include an ingredient, such as

an adjuvant, to create a stronger immune response in the subject receiving the vaccine.

[0038] In some embodiments, the DNA vaccine may be administered as a priming vaccine and a recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 may be administered subsequently as a boosting vaccine, so as to provide a combination therapy being used in a heterologous prime-boost regimen to enhance a subject's immune responses against HPV-associated diseases. For example, the DNA vaccine may be administered at a dose ranging from 100 micrograms per subject to 20 milligrams per subject, and the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 may be administered at a dose ranging from 1×10^4 pfu to 2×10^8 pfu.

[0039] In some embodiments, the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 may be TA-HPV. TA-HPV is a recombinant vaccinia viral vaccine expressing oncogenes E6 and E7 of HPV types 16 and 18. The HPV-16 and HPV-18 oncogenes E6 and E7 may be inserted in a head-to-head orientation under the control of the p7.5 and H6 promoters at a neutral site in the vaccinia virus Wyeth strain genome (L. K. Borysiewicz *et al.*, *Lancet.*, 1996 Jun 1;347(9014):1523-7). For both the HPV-16 and HPV-18 genes, the E6 termination codon may be altered to create an E6/E7 fused open reading frame and defined mutation introduced to inactivate the Rb-binding site in E7. The TA-HPV may be administered at a dose ranging from 1×10^4 pfu to 2×10^8 pfu. The TA-HPV may be administered preferably at a dose ranging from 2×10^4 pfu to 5×10^7 pfu.

[0040] Examples of the DNA constructs of the DNA vaccines are described as follows. FIG. 1 illustrates four different DNA constructs (pBI-1, pBI-10.1, pBI-11, and pBI-12). Each DNA construct includes a fusion gene that includes a subsequence encoding a signal peptide (denoted as "S" in FIG. 1), a subsequence encoding an immunostimulant (e.g., HSP70 in FIG. 1), and a subsequence encoding one or more HPV antigens (e.g., HPV-16 E7 (or E7₍₁₆₎ in FIG. 1), HPV-18 E7 (or E7₍₁₈₎ in FIG. 1), HPV-16 E6 (or E6₍₁₆₎ in FIG. 1), and/or HPV-18 E6 (or E6₍₁₈₎ in FIG. 1)). In some embodiments, the immunostimulant may include, but is not limited to, a 70 kilodalton

heat shock protein (HSP70) or a human calreticulin (CRT) protein.

[0041] In some embodiments, pBI-1, which has a nucleotide sequence set forth in SEQ ID NO: 8, is a DNA construct including a fusion gene which includes: a subsequence encoding a signal peptide, an HPV subsequence encoding a “detox” form of HPV-16 E7 expressing gene, and a subsequence encoding an HSP70. The term “detox” means the HPV oncogenes (e.g., E6 or E7) DNA are modified by mutation to express proteins that are incapable of oncogenic transformation. In some embodiments, pBI-10.1, which has a nucleotide sequence set forth in SEQ ID NO: 7, is a DNA construct including a fusion gene which includes: a subsequence encoding a signal peptide; an HPV subsequence encoding a “detox” form of HPV-16 E7 expressing gene, a “detox” form of HPV-18 E7 expressing gene, a “detox” form of HPV-16 E6 expressing gene, and a “detox” form of HPV-18 E6 expressing gene; and a subsequence encoding an HSP70.

[0042] To increase the expression of HPV antigens and further achieve DNA vaccines against HPV-associated diseases with safety and improved immunogenicity, optimized HPV subsequences respectively encoding HPV-16 E6, HPV-16 E7, HPV-18 E6, and HPV-18 E7 may be designed by mutation to express proteins that are incapable of oncogenic transformation and by codon optimization. The optimized HPV-16 E6 expressing gene may include a nucleotide sequence set forth in SEQ ID NO: 1. The optimized HPV-16 E7 expressing gene may include a nucleotide sequence set forth in SEQ ID NO: 2. The optimized HPV-18 E6 expressing gene may include a nucleotide sequence set forth in SEQ ID NO: 3. The optimized HPV-18 E7 expressing gene may include a nucleotide sequence set forth in SEQ ID NO: 4.

[0043] DNA constructs may be designed to include a fusion gene which may include an optimized HPV subsequence encoding at least one HPV antigen selected from a group consisting of: an HPV-16 E6 expressing gene set forth in SEQ ID NO: 1, an HPV-16 E7 expressing gene set forth in SEQ ID NO: 2, an HPV-18 E6 expressing gene set forth in SEQ ID NO: 3, and an HPV-18 E7 expressing gene set forth in SEQ ID NO: 4. In some embodiments, pBI-11, which has a

nucleotide sequence set forth in SEQ ID NO: 5, is a DNA construct including a fusion gene which includes: a subsequence encoding a signal peptide; an optimized HPV subsequence encoding an HPV-16 E7 expressing gene set forth in SEQ ID NO: 2, an HPV-18 E7 expressing gene set forth in SEQ ID NO: 4, an HPV-16 E6 expressing gene set forth in SEQ ID NO: 1, and an HPV-18 E6 expressing gene set forth in SEQ ID NO: 3; and a subsequence encoding an HSP70. In some embodiments, pBI-12, which has a nucleotide sequence set forth in SEQ ID NO: 6, is a DNA construct comprising a fusion gene which includes: an optimized signal sequence encoding a signal peptide; an optimized HPV subsequence encoding an HPV-16 E7 expressing gene set forth in SEQ ID NO: 2, an HPV-18 E7 expressing gene set forth in SEQ ID NO: 4, an HPV-16 E6 expressing gene set forth in SEQ ID NO: 1, and an HPV-18 E6 expressing gene set forth in SEQ ID NO: 3; and a subsequence encoding an HSP70.

Example 1: Design and synthesis of HPV DNA vaccine constructs

[0044] The pBI-1 DNA construct has been described previously as pNGVL4a-SigE7(detox)HSP70 (C. Trimble *et al. Vaccine* 2003, 21:4036–4042). In one example, the pBI-10.1 DNA construct was derived by Gibson assembly of a DNA fragment synthesized by Bio Basic (Markham, ON, Canada) encoding a fusion protein of the signal peptide, HPV-16 E7 (detox), HPV-18 E7 (detox), HPV-16 E6 (detox), and HPV-18 E6 (detox) as well as a 5' portion of HSP70 (up to the Tth111I site), flanked 5' by anEcoRI and Kozak site and 3' with a Tth111I site. The synthesized DNA fragment was cloned into the pBI-1 to replace the fragment between EcoRI and Tth111I in frame with HSP70.

[0045] Similar to pBI-10.1, the pBI-11 DNA construct included a synthesized DNA fragment encoding a fusion protein of the signal peptide, HPV-16 E7, HPV-18 E7, HPV-16 E6, and HPV-18 E6, and HSP70, but the expressing genes for HPV-16 E7, HPV-18 E7, HPV-16 E6, and HPV-18 E6 in pBI-11 were further codon-optimized. The pBI-12 DNA construct also included a synthesized DNA fragment encoding a fusion protein of the signal peptide, HPV-16

E7, HPV-18 E7, HPV-16 E6, and HPV-18 E6, and HSP70, but the expressing genes for the signal peptide, HPV-16 E7, HPV-18 E7, HPV-16 E6, and HPV-18 E6 in pBI-12, were further codon-optimized. The synthesized DNA fragment of pBI-11 or pBI-12 was cloned into the pBI-1 to replace the fragment between EcoRI and Tth111I in frame with HSP70.

[0046] The genes in the synthesized DNA fragment of each DNA construct that have been either optimized for gene expression (FIG. 1, fragments 101), mutated for detox (FIG. 1, fragments 102), or based on native papillomaviral sequences (FIG. 1, fragments 103), are shown in FIG. 1.

Example 2: HPV antigen expression level is enhanced through codon optimization

[0047] To determine whether cells transfected with the various DNA constructs appropriately produced the encoded antigen, western blot analysis was conducted. HEK 293 cells were transfected with 10 micrograms (μg) of one of pBI-1, pBI-10.1, pBI-11, or pBI-12 DNA constructs. After 48 hours of transfection, cell lysates were collected for western blot analysis, the results of which are illustrated in FIG. 2. pBI-1, pBI-10.1, pBI-11 or pBI-12 was separated on Precast Tris-HCl protein gel (Life Technology, Rockville, MD, USA) and then transferred on to nitrocellulose membrane (Bio-Rad[®] Laboratories, Hercules, CA). After blocking, the membrane was hybridized with anti-HPV-16-E7 monoclonal antibody (denoted as HPV16 E7 ab in FIG. 2) (8C9 clone from Invitrogen, Thermo Fisher Scientific, Waltham, MA), anti-HPV-18/16-E6 (denoted as HPV18/16 E6 ab in FIG. 2) (CIP5 clone from Abcam PLC, Cambridge, UK) or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (denoted as GAPDH in FIG. 2) (catalog number [no.] 60004-1-Ig; Proteintech[®] Group, Rosemont, IL) to characterize the expression of the fusion protein containing HPV-16 E7, the fusion protein containing HPV-16/18 E6, or GAPDH (Cat:60004-1-Ig, Proteintech[®] Group, Rosemont, IL) as loading control. Antibody binding was detected using a peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham[™], Piscataway, NJ, USA) and chemiluminescence

(ECL⁺ detection kit; AmershamTM, Piscataway, NJ, USA).

[0048] As indicated in the results shown in FIG. 2, all the DNA constructs showed the expression of a fusion protein that contains HPV-16 E7 antigen. Compared to the cells transfected with pBI-10.1, those cells transfected with pBI-11 and pBI-12 resulted in a higher HPV-16 E7 expression level, suggesting that the HPV-16 E7 expression level was successfully enhanced by the optimized HPV subsequence in pBI-11 and pBI-12.

[0049] Furthermore, HPV-16 E6 and/or HPV-18 E6 were detected in the cells transfected with either pBI-10.1, pBI-11 or pBI-12. In comparison, HPV-16 E6 and/or HPV-18 E6 expression levels in the cells transfected with pBI-11 and pBI-12 were higher than those in the cells transfected with pBI-10.1, indicating the optimized HPV subsequence of pBI-11 and pBI-12 enabled the cells to express HPV-16 E6 and HPV-18 E6 in an increased amount.

[0050] Taken together, the results suggested that the optimized HPV subsequences in pBI-11 and pBI-12 achieved an elevated expression level of HPV antigen.

Example 3: Cells transfected with pBI-11 or pBI-12 are able to enhance presentation of HPV antigenic peptide by MHC class I molecules

[0051] To compare the capacity of HPV antigen presentation by cells transfected with either pBI-10.1, pBI-11, or pBI-12 DNA construct, *in vitro* T cell activation assays were performed.

[0052] 293-D^b or 293 K^b cells were transfected with either pBI-10.1, pBI-11, or pBI-12 DNA construct or mock transfected using Lipofectamine 2000. 24 hours later, these cells were harvested and cocultured with either murine H-2D^b-restricted HPV-16 E7 (aa 49-57) peptide-specific CD8⁺ T cells (as depicted in FIG. 3A) or murine H-2K^b-restricted HPV-18 E6 (aa 67-75) peptide-specific CD8⁺ T cells (as depicted in FIG. 3B) in the presence of GolgiPlugTM (BD Biosciences, San Diego, CA). The cells were then harvested, and IFN- γ intracellular staining was performed to determine the activation of HPV-16 E7 or HPV-18 E6 antigen-specific CD8⁺ T cells.

[0053] As indicated in the results shown in FIGS. 3A and 3B, cells transfected with pBI-11 or pBI-12 were able to activate more HPV-16 E7 peptide (aa 49-57)-specific CD8⁺ T cells and HPV-18 E6 peptide (aa 67-75)-specific CD8⁺ T cells than cells transfected with pBI-10.1. Mock transfected cells did not generate any appreciable activation of HPV antigenic peptide (e.g., both E7 peptide (aa 49-57)-specific CD8⁺ T cells and E6 peptide (aa 67-75)-specific CD8⁺ T cells). Accordingly, the data showed that the enhanced expression of HPV-16/18 E6/E7 fusion protein in the cells transfected with pBI-11 or pBI-12 facilitated the presentation of HPV antigens by major histocompatibility complex (MHC) class I molecules to activate HPV antigen-specific CD8⁺ T cells.

Example 4: pBI-11 and pBI-12 lead to enhanced HPV-16 E7-specific CD8⁺ T cell-mediated immune responses

[0054] *In vivo* T cell activation assays were performed to compare immune response in mice vaccinated with either pBI-10.1, pBI-11, or pBI-12.

[0055] FIG. 4A is a schematic illustration of the experiment design. 6- to 8-week-old female C57BL/6 mice purchased from Taconic Biosciences (Germantown, NY) were vaccinated with 25 micrograms (μg)/mouse of either pBI-1, pBI-10.1, pBI-11, or pBI-12 DNA construct intramuscularly 3 times at 7-day intervals. One week after the last vaccination, splenocytes were collected for analysis of CD8⁺ T cell response as determined by intracellular cytokine staining for interferon-γ followed by flow cytometry after stimulation with the known MHC-I peptides in HPV-16 E6 and E7 and HPV-18 E6.

[0056] As indicated in the results shown in FIG. 4B, both pBI-11 and pBI-12-vaccinated mice showed significantly higher E7-specific CD8⁺ T cell-mediated immune responses than pBI-10.1-vaccinated mice. That is, pBI-11 and pBI-12, which include the optimized HPV subsequence, could induce enhanced antigen-specific CD8⁺ T cell-mediated immune response *in vivo*.

[0057] Based on the fusion protein expression levels and the data of *in vivo* T cell activation assay, it is suggested that the enhanced expression of HPV antigen in cells transfected with pBI-11 or pBI-12, compared to that in cells transfected with pBI-10.1, due to codon optimization contributes to more potent T cell-mediated immune responses to the HPV antigens in the vaccinated mice.

Example 5: pBI-11 DNA construct elicits a therapeutic antitumor response in an HPV-16 E6/E7 expression tumor model, TC-1

[0058] The ability of the DNA construct of the present disclosure to generate therapeutic antitumor effects against HPV-associated diseases were examined in the HPV-16 E6/E7⁺ TC-1 tumor model.

[0059] FIG. 5A is a schematic illustration of the experiment design. 6- to 8-week-old female C57BL/6 mice were injected with 2×10^5 of TC-1 tumor cells subcutaneously on day 0. On days 3, 6, and 10, the tumor-bearing mice were either vaccinated with pBI-10.1, pBI-11 or pBI-12 (25 μ g/50 microliters (ul)/mouse) through intramuscular (I.M.) injection or left untreated as a control. In some embodiments, alternative and/or additional vaccination, such as TA-HPV, could be used to vaccinate the mice as the boosting vaccine described herein.

[0060] One week after final vaccination, PBMCs were collected for tetramer staining. For tetramer staining, mouse PBMCs were stained with purified anti-mouse CD16/32 first and then stained with FITC-conjugated anti-mouse CD8a and PE-conjugated HPV-16 E7 (aa 49-57) peptide-loaded H-2D^b tetramer or PE-conjugated HPV-18 E6 (aa 67-75) peptide-loaded H-2K^b tetramer at 4°C for 1 hour. After washing, the cells were stained with 7-AAD. The cells were acquired with the FACSCaliburTM flow cytometer and analyzed with CellQuest Pro software (BD Biosciences, Mountain View, CA). The results of tetramer staining are shown in FIGS. 5B and 5C.

[0061] The growth of the tumor was monitored twice a week by palpation and digital caliper

measurement. Tumor volume was calculated using the formula $[\text{largest diameter} \times (\text{perpendicular diameter})^2] \times 3.14/6$ and is shown in FIG. 5D. The survival rate of the tumor-bearing mice was recorded, as illustrated in FIG. 5E, where both natural death and a tumor diameter greater than 2 cm leading to death were counted as death.

[0062] FIGS. 5B and 5C demonstrate that, compared to the untreated mice, tumor-bearing mice treated with pBI-10.1 showed HPV-16 E7-specific and HPV-18 E6-specific CD8⁺ T cell-mediated immune responses. Moreover, tumor-bearing mice treated with pBI-11 or pBI-12 further exhibited significantly higher HPV-16 E7-specific and HPV-18 E6-specific CD8⁺ T cell-mediated immune responses than mice vaccinated with pBI-10.1. Additionally, as shown in FIG. 5C, tumors in mice vaccinated with pBI-11 or pBI-12 grew significantly slower than in mice treated with pBI-10.1. Furthermore, vaccination with pBI-11 or pBI-12 also resulted in better survival than that for mice treated with pBI-10.1 (FIG. 5D). As a result, the present disclosure of the DNA vaccine including a DNA construct which includes a fusion gene having an optimized HPV subsequence, such as pBI-11 and pBI-12, can effectively enhance antitumor immune response against established TC-1 tumors and prolong survival of the tumor-bearing mice.

[0063] In conclusion, DNA vaccines having the optimized HPV subsequence encoding HPV-16 E6 (SEQ ID NO: 1), HPV-16 E7 (SEQ ID NO: 2), HPV-18 E6 (SEQ ID NO: 3), and/or HPV-18 E7 (SEQ ID NO: 4) of the present disclosure significantly induce enhanced HPV antigen expression and improved immune responses compared to DNA vaccines without the optimized HPV subsequence. It is surprising that the optimized HPV subsequence of the present disclosure successfully achieves a stronger antigen-specific immune response *in vivo* and further translates into more potent antitumor efficacy.

[0064] Those skilled in the art will readily observe that numerous modifications and alterations of the device and method may be made while retaining the teachings of the disclosure. Accordingly, the above disclosure should be construed as limited only by the metes and bounds of the appended claims.

What is claimed is:

1. A DNA vaccine, comprising:
a DNA construct comprising a fusion gene,
wherein the fusion gene includes an optimized human papillomavirus (HPV) subsequence encoding at least one HPV antigen, and
wherein the optimized HPV subsequence comprises one or more of:
an HPV-16 E6 expressing gene set forth in SEQ ID NO: 1,
an HPV-16 E7 expressing gene set forth in SEQ ID NO: 2,
an HPV-18 E6 expressing gene set forth in SEQ ID NO: 3, and
an HPV-18 E7 expressing gene set forth in SEQ ID NO: 4.
2. The DNA vaccine according to claim 1,
wherein the fusion gene further comprises a subsequence encoding an immunostimulant.
3. The DNA vaccine according to claim 2,
wherein the immunostimulant is a 70 kilodalton (kDa) heat shock protein (HSP70) or a human calreticulin (CRT) protein.
4. The DNA vaccine according to claim 2,
wherein the optimized HPV subsequence comprises the HPV-16 E6, HPV-16 E7, HPV-18 E6, and HPV-18 E7 expressing genes, and
wherein the fusion gene comprises SEQ ID NO: 5.
5. The DNA vaccine according to claim 2,
wherein the fusion gene further comprises an optimized signal sequence,
wherein the fusion gene comprises SEQ ID NO: 6.
6. Use of the DNA vaccine according to any one of claims 1-5 in the manufacture of a medicament for the treatment of an HPV-associated disease in a subject in need thereof.
7. Use of a combination of (a) the DNA vaccine according to any one of claims 1-5; and (b) a recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 in the

manufacture of a medicament for the treatment of an HPV-associated disease in a subject in need thereof, wherein the DNA vaccine is for administration to the subject as a priming vaccine and the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 is for administration to the subject as a boosting vaccine.

8. The use according to claim 7, wherein the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 is TA-HPV.

9. The use according to claim 8,
wherein the TA-HPV is administered at a dose ranging from 1×10^4 plaque-forming units (pfu) to 2×10^8 pfu.

10. The use according to claim 8,
wherein the DNA vaccine is administered at a dose ranging from 100 micrograms per subject to 20 milligrams per subject.

11. A DNA vaccine according to any one of claims 1-5 for use in the treatment of an HPV-associated disease in a subject in need thereof.

12. A combination of (a) the DNA vaccine according to any one of claims 1-5; and (b) a recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 for use in the treatment of an HPV-associated disease in a subject in need thereof, wherein the DNA vaccine is for administration to the subject as a priming vaccine and the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 is for administration to the subject as a boosting vaccine.

13. The combination for use according to claim 12, wherein the recombinant vaccinia virus expressing E6 and E7 of both HPV-16 and HPV-18 is TA-HPV.

14. The combination for use according to claim 13,
wherein the TA-HPV is administered at a dose ranging from 1×10^4 plaque-forming units (pfu) to 2×10^8 pfu.

15. The combination for use according to claim 13,

wherein the DNA vaccine is administered at a dose ranging from 100 micrograms per subject to 20 milligrams per subject.