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(71) Applicant: **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 3400 N. Charles Street, Baltimore, Maryland 21218 (US).

(72) Inventors: **WU, T.C.**; 11002 Nacirema Lane, Stevenson, Maryland 21153 (US). **HUNG, Chien-Fu**; 13 Ballyhean Court, Timonium, Maryland 21093 (US). **RODEN, Richard**; 13 Luna Lane, Severna Park, Maryland 21146 (US). **TRIMBLE, Cornelia L.**; 7 Club Road, Baltimore, Maryland 21210 (US).

(74) Agent: **CONTRERA, Joseph**; 100 N. Charles Street, 5th Floor, Baltimore, Maryland 21201 (US).

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(54) Title: TREATMENT REGIMEN USING CANCER VACCINES AND LOCAL ADJUVANTS AND THEIR USE

(57) Abstract: The present invention provides compositions and methods for locally administering an adjuvant following HPV vaccine compositions such as CRT/E7 DNA vaccine administration, on antigen-specific CD8+ T cell-mediated immune responses and antitumor effects for use in treating HPV-related disease. In some embodiments, the present invention provides compositions and methods for treatment of HPV-related disease comprising administering immunological compositions followed by administration of an adjuvant, such as the toll-like receptor 7 agonist, imiquimod.

TREATMENT REGIMEN USING CANCER VACCINES AND LOCAL ADJUVANTS AND THEIR USE

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application Nos. 61/971,598, filed on March 28, 2014, and 62/112,266, filed February 5, 2015, both of which are hereby incorporated by reference for all purposes as if fully set forth herein.

STATEMENT OF GOVERNMENTAL INTEREST

[0002] This invention was made with government support under grant nos. CA098252 and CA114425, awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 26, 2014, is named P12928-01_ST25.txt and is 2,335 bytes in size.

BACKGROUND OF THE INVENTION

[0004] Human papillomaviruses (HPVs) are the primary etiologic agents for cervical cancer and subsets of vaginal, vulvar and anal cancers and head and neck cancers. Approximately 90% of vaginal, vulvar and anal cancers associated with HPV are attributable to HPV16. Moreover, while surgical treatment is quite effective and well tolerated for pre-cancer and early cancer lesions of the cervix, a subset of treated patients are at risk for preterm delivery and/or cervical incompetence, and premature rupture of membranes (2), which can be devastating to young women. Furthermore, surgical treatment of vaginal, vulvar and anal intraepithelial lesions is associated with significant morbidity and high recurrence rates. As such, it is important to develop effective alternative therapeutic methodologies to treat HPV-associated pre-cancer lesions and cancers, particularly for the non-cervical sites that are predominantly associated with HPV16.

[0005] Immunotherapy can potentially be used to treat HPV-associated disease. HPV oncoproteins E6 and E7 are required for the induction and maintenance of cellular transformation, lack many of the concerns for immune tolerance of self-antigens, and are consistently co-expressed in all HPV-infected, but not normal cells. Therapeutic vaccines, through activation of the adaptive immune system, can elicit a specific immune response to target precancer and cancer cells while sparing normal cells. A long peptide-based therapeutic vaccine targeting HPV16 E6 and/or E7 shows partial efficacy against HPV16+ high grade VIN (NEJM, 2009;361:1838-47). These findings suggest that the viral oncoproteins have evolved to be weak antigens and therefore need approaches to render them more immunogenic and thus more potently activate HPV-specific cytotoxic T cell responses.

[0006] DNA vaccines targeting E6/E7 are promising because the antigen is produced intracellularly and can therefore enter the MHC class I pathway that is critical for the induction of cytotoxic T cell responses. To circumvent the poor immunogenicity of the HPV oncoproteins, the inventors have previously developed an approach to enhance their immune presentation upon expression from a naked DNA vector. In this therapeutic HPV DNA vaccine, HPV16 E7 was linked to calreticulin (CRT/E7), a heat shock-related chaperone protein that enhances antigen processing and directs binding to, and activation of antigen-presenting cells (J. Clin. Invest., 2001;108:669-78). Vaccination by intramuscular administration of CRT/E7 DNA generated potent systemic cell-mediated immune responses against HPV16 E7 and subcutaneous TC-1 tumor, a syngeneic model of advanced cervical cancer that expresses HPV16 E6 and E7 (J. Biomed. Sci., 2010;17:32). Furthermore, the clinical grade version of this vaccine, pNGVL4a-CRT/E7 (detox), is being tested in patients with HPV16+ high grade cervical intraepithelial neoplasia (CIN2/3) using different routes of administration.

[0007] Application of an adjuvant locally can further enhance site-specific immunity. For example, Luci et al linked the nontoxic B subunit of cholera toxin (CTB) to model antigen OVA and found that its administration intravaginally increased OVA-specific $CD8^+$ T cells in the draining lymph nodes and genital mucosa (J Immuno, 2006;176:2749-57). Additionally, Wille-Reece et al found that vaccination of non-human primates using toll-like receptor (TLR) agonists as adjuvants generated stable antigen-specific $CD8^+$ T cells (J. Exp. Med., 2006;203:1249-58).

[0008] However, there still exists a need for improved methods for treating HPV-associated cancers.

SUMMARY OF THE INVENTION

[0009] In accordance with some embodiments, the present inventors examined the effects of a locally administered adjuvant application following CRT/E7 DNA vaccine administration on antigen-specific CD8+ T cell-mediated immune responses and antitumor effects. It was found that following treatment with an adjuvant, such as the toll-like receptor 7 agonist, imiquimod, the vaginal tissue of mice exhibited a significant increase in local E7-specific CD8+ T cells. Furthermore, upon systemic adoptive transfer of E7-Luc T cells, it was found that adjuvant treatment potently attracted them to the cervicovaginal tracts of mice. The inventors also found that mice treated with CRT/E7 DNA vaccine followed by intravaginal adjuvant deposition, compared to either treatment alone, generated synergistic antitumor effects and dramatically improved survival. It was also found that the local inflammation induced by the activation of antigen-specific CD8+ T cells could recruit CD8+ T cells with specificity for other antigen to the vaginal tissue. Additionally, it was found that imiquimod application induced local CXCL9/10 expression and that the antigen-specific T cells that accumulate in the cervicovaginal tract following imiquimod application express CXCR3 as well as the Trm marker CD49a.

[0010] Therefore, in accordance with some embodiments, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering systemically to the subject a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and administering to the subject an effective amount of a composition comprising an adjuvant to the site of the HPV-related disease or infection in the subject.

[0011] In accordance with an embodiment, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering systemically to the subject a therapeutically effective amount of a vaccine composition comprising HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and administering to the subject an effective amount of a composition comprising an adjuvant to the site of the HPV-related disease or infection in the subject.

[0012] In accordance with some embodiments, the vaccine composition induces a humoral or a cellular immune response, antigen-specific or innate. The immunogenic composition of the invention can be immunogenic against 1, 2, 3, 4, 5, or more of mucosal high-risk types (e.g., HPV 16, 18, 31, 33, 45, 52, 58, 68, or 76), mucosal low-risk types (e.g. HPV 6 and 11), HPV13, 32 causing Heck's disease (focal epithelial hyperplasia of other oral mucosa), cutaneous low risk types (skin-tropic types) causing skin warts (e.g., HPV1, 2, 3, 4, 7, 10, 27, 57, etc.) and/or cutaneous beta-types (e.g. beta-type HPV5, 8, 9, 12, 14, 15, 38, etc.) of papillomaviruses, or animal papillomavirus types. With regard to the nomenclature of PV, all beta PV are cutaneous types. However, cutaneous types are usually referred to as those that induce common, palmar, plantar, or plane skin warts (these are found in the alpa, gamma, mu, nu genus). The beta types generally only induce skin warts (or skin cancer) in EV patients or immunosuppressed patients.

[0013] In accordance with some embodiments, the immunogenic vaccine composition of the invention can be effective against human papillomaviruses (e.g., against mucosal high-risk, low-risk, cutaneous and beta (e.g. beta-type HPV 5) papillomaviruses). The immunogenic composition of the invention can be formulated in a variety of manners, including a lyophilized or powdered form, a formulation for administration by inhalation, ingestion (e.g., as a pill), in a viral or bacterial vector, or as a component of a sexual lubricant. One mode of administration is similar to that of the currently existing HPV vaccines, for i.m. inoculation with local application of adjuvants as described herein. For administration in developing countries, which may lack adequate refrigeration, formulations for lyophilized, inhalation, ingestion, or viral or bacterial vectors may be more suitable.

[0014] Thus, in accordance with an embodiment, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering to the subject a composition comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid and subsequently administering to the subject an effective amount of a composition comprising an adjuvant to the site of the HPV-related disease or infection in the subject.

[0015] In accordance with another embodiment, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering to the subject a composition comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid and subsequently administering to the subject an effective amount of a composition comprising

an toll-like receptor 7 agonist to the site of the HPV-related disease or infection in the subject.

[0016] In accordance with a further embodiment, the present invention provides a treatment regimen for inducing the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract of a subject infected with HPV in the cervicovaginal tract, comprising administering to the subject a composition comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid and subsequently administering to the subject an effective amount of a composition comprising toll-like receptor 7 agonist to the cervicovaginal tract of the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figures 1A-1E illustrate that the deposition of imiquimod in the vagina attracts $CD8^+$ T cells to the genital tract. Groups of mice were vaccinated twice with a DNA plasmid encoding pNGVL4a-CRT/E7 (40μg) by intramuscular injection. Mice were treated with or without imiquimod. Blood was drawn one week after vaccination and cells were stained with anti-CD8 antibody, H2-D^b E7 tetramer, and 7-AAD (to exclude dead cells). (1A) Representative flow cytometry analysis showing E7-specific CD8+ T cells in peripheral blood. (1B) Bar graph depicting the percentage of E7-specific CD8+ T cells in circulation. (1C) PBS or imiquimod 5% cream was applied in the vaginal tract one week after systemic vaccination with pNGVL4a-CRT/E7(detox). Genital tract tissue was excised and digested. Bar graph shows the absolute number of CD8+ T cells in the genital tracts of treated mice. (1D) Representative flow cytometry. (1E) Bar graph depicting the absolute number of E7-specific CD8+ T cells in the genital tracts of mice treated with or without imiquimod (*$p<0.05$, **$p<0.01$).

[0018] Figures 2A-2C show that the systemic vaccination combined with local imiquimod application generated potent antitumor effects in the vaginal tract. (2A) Tumor challenge and vaccination/imiquimod application schedule. Mice were treated with pNGVL4a-CRT/E7(detox) vaccine alone (VA), imiquimod alone (IM), or both (VA+IM). (2B) Bioluminescence imaging showing the TC-1 tumors following challenge with TC-1-Luc cells ($5x10^4$/mouse) administered in the vagina. (2C) Bar graph depicting bioluminescence intensity of the various treatment groups over time (**$p<0.01$).

[0019] Figures 3A-3D depict antigen-experienced Trm cell accumulation in the vaginal area after antigen stimulation. (3**A**) Illustration of assay. Briefly, E7 (RAHYNIVTF) (SEQ ID NO: 1) peptide or PBS was administered locally followed by adoptive transfer of CFSE-labeled OT-1 CD8+ T cells. Two days after adoptive transfer, PBMCs and vaginal tissue were examined for CFSE labeled OT-1 CD8+ T cells. (3**B**) Representative flow cytometry. (3**C**) Bar graph depicting the number of CFSE+ cells in the spleens of treated mice. (3**D**) Bar graph depicting the percentage of CFSE+ cells in the genital tracts of treated mice (*$p<0.05$; **$p<0.01$).

[0020] Figures 4A-4B show the expression of CXCL9 and CXCL10 following imiquimod treatment and the characterization of the CD8+ T cell accumulation in the cervicovaginal tract. (4**A**) Expression levels of TLR7, IFNγ, CXCL9, and CXCL10 were evaluated from mRNA extracted from vaginal tissue treated with PBS or imiquimod by qRT-PCR. Gene expression levels were normalized to β-Actin housekeeping gene, and data were represented as fold differences. (4**B**) Surface integrin markers of cervicovaginal CD8+ T cells were screened (*$p<0.05$; **$p<0.01$).

[0021] Figures 5A-5E depict recruitment of T cells from circulation to treatment location in IFNγR-/- mice. (5A) mRNA was extracted from the imiquimod-treated vaginal tissue of Wt mice and IFNγR -/- mice. The expression levels of TLR7, IFNγ, CXCL9, and CXCL10 were detected by qRTPCR and are depicted in the bar graph. (5B) Bar graph showing the number of E7-specfic CD8+ T cells per 3x105 PBMCs. (5C) Total number of CD8+ T cells in the genital tracts of mice. (5D) Representative flow cytometry and (5E) bar graph of E7-specific CD8+ T cells in vaginal tissue (*p<0.05; **p<0.01).

[0022] Figures 6A-6B show the characterization of CD8+ T cell migration in CXCR3 knockout mice following vaginal deposition of imiquimod. Wild type and CXCR3 knockout mice were vaccinated with pNGVL4a-CRT/E7(detox) and treated with imiquimod in the cervicovaginal tract. (6A) Bar graph showing the absolute number of CD8+ T cells number in the cervicovaginal tracts of Wt and CXCR3-/- mice. (6B) Representative flow cytometry analysis of E7-specific CD8+ T cells in the cervicovaginal tracts of Wt and CXCR3-/- mice.

[0023] Figures 7A-7B (Supplemental Figure 1). E7-specific T cells efficiently migrated from circulation to the genital tract following imiquimod application. PBS or imiquimod was applied to the vaginal areas of naïve mice one day prior to adoptive transfer of luciferase-expressing E7-specific CD8+ T cells ($5x10^5$/mouse). The genital tracts were excised 48 hours after adoptive transfer and the bioluminescence signal intensity was examined. (7A)

Representative imaging. (7B) Bar graph depicting the bioluminescence intensity of the genital tracts of treated mice (*p<0.05).

[0024] Figures 8A-8C (Supplemental Figure 2). Characterization of tumor infiltrating T cells following treatment with systemic DNA vaccination and locally applied imiquimod. Mice were challenged with TC-1 tumor cells intramucosally, generating an orthotopic murine cervical cancer model. Groups of mice were treated with DNA vaccination alone (VA), imiquimod alone (IM), or with both treatments (VA+IM). (8A) Survival of the various groups of mice over time. (**p<0.01). (8B) Tumors were excised and examined for tumor infiltrating lymphocytes on day 21 using flow cytometry analysis. Bar graph depicts the total number of $CD8^+$ T cells in tumor loci. (8C) Bar graph depicting the number of E7-specific CD8+ T cells in tumor loci (*p<0.05; **p<0.01).

[0025] Figures 9A-9D (Supplemental Figure 3). Characterization of systemic and local E7-specific CD8+ T cell in mice treated with N9. PBS or N9 was applied in the cervicovaginal tract one week after systemic DNA vaccination. Blood was drawn and genital tract tissue was excised and digested. (9A) Representative flow cytometry analysis showing E7-specific CD8+ T cells in peripheral blood. (9B) Bar graph depicting the percentage of E7-specific CD8+ T cells in circulation. (9C) Representative flow cytometry analysis showing E7-specific CD8+ T cells in the cervicovaginal tract. (9D) Bar graph depicting the percentage of E7-specific CD8+ T cells in the cervicovaginal tract.

DETAILED DESCRIPTION OF THE INVENTION

[0026] Therefore, in accordance with some embodiments, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering systemically to the subject a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and administering to the subject an effective amount of a composition comprising an adjuvant to the site of the HPV-related disease or infection in the subject.

[0027] In accordance with an embodiment, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering systemically to the subject a therapeutically effective amount of a vaccine composition comprising HPV associated proteins selected from

the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and administering to the subject an effective amount of a composition comprising an adjuvant to the site of the HPV-related disease or infection in the subject.

[0028] In accordance with another embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine composition comprising HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered systemically to the subject and wherein the adjuvant is administered to the subject to the site of the HPV-related disease or infection in the subject.

[0029] In accordance with a further embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered to the subject at the site of the HPV-related disease or infection in the subject and wherein the adjuvant is administered to the subject at the site of the HPV-related disease or infection in the subject.

[0030] In some embodiments, the vaccine composition is administered to the subject before administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

[0031] In some other embodiments, the vaccine composition is administered to the subject concurrently with administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

[0032] In accordance with certain other embodiments, the vaccine composition is administered to the subject for repeated dose intervals, and then followed by administration of the adjuvant to the site of infection for one or more dose intervals.

[0033] In accordance with some embodiments, the immunogenic composition induces a humoral or a cellular immune response, antigen-specific or innate. The immunogenic composition of the invention can be immunogenic against 1, 2, 3, 4, 5, or more of mucosal high-risk types (e.g., HPV 16, 18, 31, 33, 45, 52, 58, 68, or 76), mucosal low-risk types (e.g.

HPV 6 and 11), HPV13, 32 causing Heck's disease (focal epithelial hyperplasia of other oral mucosa), cutaneous low risk types (skin-tropic types) causing skin warts (e.g., HPV1, 2, 3, 4, 7, 10, 27, 57, etc.) and/or cutaneous beta-types (e.g. beta-type HPV5, 8, 9, 12, 14, 15, 38, etc.) of papillomaviruses, or animal papillomavirus types. With regard to the nomenclature of PV, all beta PV are cutaneous types. However, cutaneous types are usually referred to as those that induce common, palmar, plantar, or plane skin warts (these are found in the alpa, gamma, mu, nu genus). The beta types generally only induce skin warts (or skin cancer) in EV patients or immunosuppressed patients.

[0034] In accordance with an embodiment, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering to the subject a composition comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid and subsequently administering to the subject an effective amount of a composition comprising an adjuvant to the site of the HPV-related disease or infection in the subject.

[0035] In accordance with another embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered systemically to the subject and wherein the adjuvant is administered to the subject to the site of the HPV-related disease or infection in the subject.

[0036] As used herein, the term "pNGVL4a-CRT/E7(detox) plasmid vaccine" means a DNA vaccine having DNA sequences which encode antigenic peptides, e.g., those derived from human papillomavirus (HPV), HPV-16 E7, as detailed in U.S. Patent Application No. 12/438,300, filed June 7, 2010, and incorporated by reference herein.

[0037] As used herein, the terms "protein," "polypeptide," and "peptide," as used herein, are not restricted to any particular number of amino acids; these terms are sometimes used interchangeably herein. The properties and amino acid sequences of the proteins of the invention, and of the nucleic acids encoding them, are well-known and can be determined routinely, as well as downloaded from various known databases. See, e.g., the NCBI GenBank databases. Some sequences are provided herein. This information is accurate as of the date of filing of this application. However, some sequence information is routinely updated (e.g. to correct mistakes in the previous entries), so updated (corrected) information

about the proteins and nucleic acids encoding them is included in this application. Information provided in the sequence databases discussed herein is incorporated by reference in the present application.

[0038] The human papillomavirus is a DNA tumor virus that causes epithelial proliferation at cutaneous and mucosal surfaces. More than 100 different types of the virus exist, including approximately 30 to 40 strains that infect the human genital tract. Of these, there are oncogenic or high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, and 58) that are associated with cervical, vulvar, vaginal, penile, oral, throat and anal cancers, and non-oncogenic or low-risk types (6, 11, 40, 42, 43, 44, and 54) that are associated with anogenital condyloma or genital warts. HPV 16 is the most oncogenic, accounting for almost half of all cervical cancers, and HPV 16 and 18 together account for approximately 70% of cervical cancers. HPV 6 and 11 are the most common strains associated with genital warts and are responsible for approximately 90% of these lesions.

[0039] In accordance with some embodiments the HPV-associated disease treated by the inventive compositions and methods includes atypical squamous cells that are subclassified into ASC-US (undetermined significance), and atypical glandular cells of undetermined significance (AGUS).

[0040] In accordance with an embodiment, the HPV-associated disease treated by the inventive compositions and methods is cancer, including cervical, vulvar, vaginal, penile, oral, throat and anal cancers. In some embodiments, the cancer is cervical cancer.

[0041] In accordance with some embodiments, the inventive compositions and methods comprise vaccination at the cutaneous and mucosal surfaces which are infected with HPV. In an embodiment, the mucosal tissue is the vaginal mucosa. In other embodiments, the cutaneous and mucosal surfaces are in the cervicovaginal tract.

[0042] As used herein, the term "subject" can mean a subject suspected of having cervical cancer or suspected of having an increased risk of having a cervical neoplasia and can include a patient presenting cervical intraepithelial neoplasia (CIN), and/or low grade squamous intraepithelial lesion (LSIL) and/or high grade squamous intraepithelial lesion (HSIL), or any other abnormal Pap smear or cytological test.

[0043] As used herein, the term "subject" can also mean a subject suspected of having an HPV infection or HPV related disease, and also includes a subject that has either been exposed to HPV or has evidence of infection with HPV of any variant strain.

[0044] In accordance with one or more embodiments of the present invention, it will be understood that the types of cancer diagnosis which may be made, using the methods provided herein, is not necessarily limited. For purposes herein, the cancer can be any cancer. As used herein, the term "cancer" is meant any malignant growth or tumor caused by abnormal and uncontrolled cell division that may spread to other parts of the body through the lymphatic system or the blood stream.

[0045] As used herein, the term "treat," as well as words stemming therefrom, includes diagnostic and preventive as well as disorder remitative treatment.

[0046] As used herein, the term "subject" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

[0047] The terms "treat," and "prevent" as well as words stemming therefrom, as used herein, do not necessarily imply 100% or complete treatment or prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the inventive compositions and methods can provide any amount of any level of treatment or prevention of HPV related cancer in a mammal. Furthermore, the treatment or prevention provided by the inventive compositions and method can include treatment or prevention of one or more conditions or symptoms of the disease, e.g., cancer, being treated or prevented. Also, for purposes herein, "prevention" can encompass delaying the onset of the disease, or a symptom or condition thereof.

[0048] The term “adjuvant” as used herein, generally means a substance that is added to the vaccine to increase the body's immune response to the vaccine. Adjuvants are inorganic or organic chemicals, macromolecules or entire cells of certain killed bacteria, which enhance the immune response to an antigen. They may be included in a vaccine to enhance the recipient's immune response to the supplied antigen, thus minimizing the amount of injected foreign material. In some embodiments, the adjuvants used in the methods of the present invention include toll-like receptor (TLR) ligands including the use of two or more ligands.

TLR ligands are capable of generating stronger Trm recruitment. Currently, two TLR agonists are FDA approved for use in cancer patients in addition to imiquimod, the TLR4 agonist monophosphoryl lipid A (MPL), and the TLR2/4 agonist bacillus Calmette–Guérin (BCG). Also of interest is the TLR7/8 agonist, resiquimod, which is an imidazoquinoline like imiquimod, and has been shown to have antitumor effects.

[0049] Polyinosinic-polycytidylic acid (poly(I:C)) is a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Poly(I:C) is recognized by TLR3 inducing the activation of NF-kB and the production of cytokines.

[0050] TLR9 agonists are being developed for clinical application in oncology and viral infections by Pfizer, Dynavax Technologies and GlaxoSmithKline among others. It will be understood by those of skill in the art that alternative adjuvants can be used in the inventive compositions and methods. In a specific embodiment, the adjuvant used is imiquimod (1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine), also known under the trade names Aldara and Zyclara, and by Mochida as Beselna. It is also referred to as R-837.

[0051] In accordance with some other embodiments, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering to the subject a composition comprising an immunological composition comprising one or more protein or peptide sequences encoding two or more HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2 and subsequently administering to the subject an effective amount of a composition comprising a toll-like receptor 7 agonist to the site of the HPV-related disease or infection in the subject.

[0052] In accordance with another embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered systemically to the subject and wherein the adjuvant is administered to the subject to the site of the HPV-related disease or infection in the subject.

[0053] In accordance with another embodiment, the present invention provides a treatment regimen for generating an immune response against human papillomavirus (HPV)-associated disease in a subject comprising administering to the subject a composition

comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid and subsequently administering to the subject an effective amount of a composition comprising a toll-like receptor 7 agonist to the site of the HPV-related disease or infection in the subject.

[0054] In accordance with another embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid, and an effective amount of a composition comprising toll-like receptor 7 agonist, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered systemically to the subject and wherein toll-like receptor 7 agonist is administered to the subject to the site of the HPV-related disease or infection in the subject.

[0055] In accordance with a further embodiment, the present invention provides a treatment regimen for inducing the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract of a subject infected with HPV in the cervicovaginal tract, comprising administering to the subject a composition comprising an immunological composition comprising one or more protein or peptide sequences encoding two or more HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2 and subsequently administering to the subject an effective amount of a composition comprising a toll-like receptor 7 agonist to the site of the HPV-related disease or infection in the subject.

[0056] In accordance with another embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in inducing the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract of a subject infected with HPV in the cervicovaginal tract, wherein the vaccine composition is administered systemically to the subject and wherein the adjuvant is administered to the subject to the site of the HPV-related disease or infection in the subject.

[0057] In accordance with a further embodiment, the present invention provides a treatment regimen for inducing the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract of a subject infected with HPV in the cervicovaginal tract, comprising administering to the subject a composition comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid and subsequently administering to the subject an

effective amount of a composition comprising a toll-like receptor 7 agonist to the cervicovaginal tract of the subject.

[0058] In accordance with another embodiment, the present invention provides a composition comprising a therapeutically effective amount of a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid, and an effective amount of a composition comprising a toll-like receptor 7 agonist, for use in inducing the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract of a subject infected with HPV in the cervicovaginal tract, wherein the vaccine composition is administered systemically to the subject and wherein the toll-like receptor 7 agonist is administered to the subject to the site of the HPV-related disease or infection in the subject.

[0059] As used herein, the term "generating an immune response against human papillomavirus (HPV)-associated disease" means the accumulation of antigen-specific CD8+ T cells in the site of infection or disease. The term can also include the migration of CXCR3+ CD8+ T cells to the site of infection or disease.

[0060] In accordance with the inventive compositions and methods, it was observed that IFNγ is essential for the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract (see Figure 5). In addition, it was observed that CXCL9/10 are upregulated following imiquimod application (Figure 4).

[0061] Previously, it has been shown that multiple local treatments with the spermicide N9 can induce inflammation in the cervicovaginal tract of mice as a result of type 1 interferon-mediated NF-κB activation. Such inflammatory events typically lead to an increase in the expression of numerous other pro-inflammatory cytokines as well as chemokines, which may assist in recruiting immune cells to the cervicovaginal tract. In one case, increase in the local production the chemokine CCL2 correlated with enhanced recruitment of inflammatory macrophages. Interestingly, here using the inventive compositions and methods, it is shown that local treatment with N9 generates a higher number of antigen-specific CD8+ T cells in the cervicovaginal tract after systemic vaccination. This may also be the result of increased inflammation induced by the N9 treatment, and the inflammatory mediators responsible for the local accumulation in T cells warrant further examination. These observations also have translational relevance as N9-based spermicides are readily available over-the-counter and in accordance with these inventive compositions and methods, could be utilized in combination with systemic HPV

vaccination to enhance their therapeutic effects against cervicovaginal intraepithelial neoplasia by application in the genital tract.

[0062] In accordance with the inventive compositions and methods, it was found that the antigen-specific T cells accumulating in the cervicovaginal tract expressed the T cell homing marker CD49a after intravaginal imiquimod application (Figure 4B). CD49a is a mucosal integrin whose expression on E7-specific CD8+ T cells has been shown to be induced following intranasal mucosal vaccination. Furthermore, CD49a was found to be crucial for intratumoral infiltration of the antigen-specific CD8+ T cells and the efficacy of cancer vaccines against mucosal tumors. Taken together, these data show that vaccination with CRT/E7 DNA and topical imiquimod application using the inventive compositions and methods, can promote the generation of mucosal-homing E7-specific CD8+ T cells that are capable of infiltrating mucosal tumors and eliciting potent antitumor effects.

[0063] Any suitable expression system may be employed. The vectors include a DNA encoding a polypeptide or fragment of the invention, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a DNA sequence if the promoter nucleotide sequence controls the transcription of the DNA sequence. An origin of replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

[0064] Suitable host cells for expression of polypeptides include prokaryotes, yeast or higher eukaryotic cells. Mammalian or insect cells are generally preferred for use as host cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al In: Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., 1985. Cell-free translation systems could also be employed to produce polypeptides using RNAs derived from DNA constructs disclosed herein. In general, molecular biology methods referred to herein are well-known in the art and are described, e.g., in Sambrook et al., Molecular Cloning: A Laboratory Manual, current

edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & sons, New York, N.Y.

[0065] In an embodiment, the methods of the present invention can include pNGVL4a-CRT/E7(detox) vaccine in conjunction with a carrier. The carrier is preferably a pharmaceutically acceptable carrier. With respect to pharmaceutical compositions, the carrier can be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the active compound(s), and by the route of administration. The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those skilled in the art and are readily available to the public. It is preferred that the pharmaceutically acceptable carrier be one which is chemically inert to the active agent(s) and one which has no detrimental side effects or toxicity under the conditions of use.

[0066] In accordance with an embodiment of the treatment regimen, the vaccine composition comprises a prime-boost combination of a fusion protein HPV6, HPV16 and/or HPV18 E6 and/or E7 and a polynucleotide expressing HPV16 and/or HPV18 E6 and/or E7.

[0067] In accordance with another embodiment of the treatment regimen, the vaccine composition comprises a prime-boost combination of polynucleotide expressing HPV16 and/or HPV18 E6 and/or E7 and a recombinant vaccinia virus expressing HPV16 and HPV18 E6 and E7.

[0068] In accordance with another embodiment of the treatment regimen, the vaccine composition comprises a prime-boost combination of polynucleotide expressing HPV16 and/or HPV18 E6 and/or E7 and a fusion protein HPV6, HPV16 and/or HPV18 E6 and/or E7.

[0069] Thus, in an embodiment, the present invention provides the use of a pharmaceutical composition comprising a vaccine construct, and an effective amount of a pharmaceutical composition comprising an adjuvant and a pharmaceutically acceptable carrier, as a medicament, preferably as a medicament for the treatment of an HPV-related disease or infection in a subject.

[0070] In another embodiment, the present invention provides the use of a pharmaceutical composition comprising a vaccine construct consisting of pNGVL4a-CRT/E7(detox) plasmid, and an effective amount of a pharmaceutical composition comprising an adjuvant and a pharmaceutically acceptable carrier, as a medicament, preferably as a medicament for the treatment of an HPV-related disease or infection in a subject.

[0071] The choice of carrier will be determined in part by the chemical properties of vaccine as well as by the particular method used to administer vaccine. Accordingly, there are a variety of suitable formulations of the pharmaceutical composition of the invention. The following formulations for parenteral, subcutaneous, intravenous, intramuscular, intraarterial, intrathecal, and intraperitoneal administration are exemplary and are in no way limiting. More than one route can be used to administer the vaccine, and in certain instances, a particular route can provide a more immediate and more effective response than another route.

[0072] Injectable formulations are in accordance with the present invention. The requirements for effective pharmaceutical carriers for injectable compositions are well-known to those of ordinary skill in the art (see, e.g., *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Trissel, 14th ed., (2007)).

[0073] For purposes of the invention, the amount or dose of pNGVL4a-CRT/E7(detox) vaccine administered should be sufficient to effect, e.g., a therapeutic or prophylactic response, in the subject over a reasonable time frame. The dose will be determined by the efficacy of the pNGVL4a-CRT/E7(detox) vaccine and the condition of a human, as well as the body weight of a human to be treated.

[0074] The preparation of vaccines that contain polypeptide or peptide sequence(s) as active ingredients is generally well understood in the art, as exemplified by U.S. Pat. Nos. 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all of which are incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions: solid forms suitable for solution in or suspension in liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants that enhance the effectiveness of the vaccines. In specific embodiments, vaccines are formulated with a combination of substances, as described in U.S. Pat. Nos. 6,793,923 and 6,733,754, which are incorporated herein by reference.

[0075] Vaccines may be administered by inhalation. In certain embodiments a vaccine can be administered as an aerosol. As used herein the term “aerosol” or “aerosolized

composition" refers to a suspension of solid or liquid particles in a gas. The terms may be used generally to refer to a composition that has been vaporized, nebulized, or otherwise converted from a solid or liquid form to an inhalable form including suspended solid or liquid drug particles. Such aerosols can be used to deliver a vaccine via the respiratory system. As used herein, "respiratory system" refers to the system of organs in the body responsible for the intake of oxygen and the expiration of carbon dioxide. The system generally includes all the air passages from the nose to the pulmonary alveoli. In mammals it is generally considered to include the lungs, bronchi, bronchioles, trachea, nasal passages, and diaphragm. For purposes of the present disclosure, delivery of a vaccine to the respiratory system indicates that a drug is delivered to one or more of the air passages of the respiratory system, in particular to the lungs.

[0076] Additional formulations which are suitable for other modes of administration include suppositories (for anal or vaginal application) and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1% to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

[0077] Typically, the attending physician will decide the dosage of pNGVL4a-CRT/E7(detox) vaccine with which to treat each individual patient, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, to be administered, route of administration, and the severity of the condition being treated. In some embodiments, the amount of vaccine composition administered is in the range of 100 pg to 4000 μg, including, for example 200 pg, 500 pg, 750 pg, 1μg, 10 μg, 50 μg, 100 μg, 250 μg, 500 μg, 1000 μg, 2000 μg, 3000 μg and 4000 μg. By way of example and not intending to limit the invention, the dose of pNGVL4a-CRT/E7(detox) vaccine can be about 1 to 100 μg of pNGVL4a-CRT/E7(detox) vaccine to the subject being treated. In some embodiments, the dosage range is about 20-50 μg of pNGVL4a-CRT/E7(detox) vaccine.

[0078] The attending physician will also decide the dosage of amount of adjuvant with which to treat each individual patient, taking into consideration a variety of factors, such as

age, body weight, general health, diet, sex, to be administered, route of administration, and the severity of the condition being treated. In some embodiments the dosage range is about 100 μg to about 100 mg of imiquimod. In an embodiment, the dosage range of imiquimod is about 200 μg to about 400 μg. The formulations can vary with the route of administration.

[0079] In certain embodiments, the adjuvant is administered topically, to the site of the HPV-related disease in the subject. In a specific embodiment, the adjuvant is imiquimod, which is administered topically in a cream formulation.

[0080] An "active agent" and a "biologically active agent" are used interchangeably herein to refer to a chemical or biological compound that induces a desired pharmacological and/or physiological effect, wherein the effect may be prophylactic or therapeutic. The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of those active agents specifically mentioned herein, including, but not limited to, salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the terms "active agent," "pharmacologically active agent" and "drug" are used, then, it is to be understood that the invention includes the active agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs etc. The active agent can be a biological entity, such as a virus or cell, whether naturally occurring or manipulated, such as transformed.

[0081] The biologically active agent may vary widely with the intended purpose for the composition. The term active is art-recognized and refers to any moiety that is a biologically, physiologically, or pharmacologically active substance that acts locally or systemically in a subject. Examples of biologically active agents, that may be referred to as "drugs", are described in well-known literature references such as the Merck Index, the Physicians' Desk Reference, and The Pharmacological Basis of Therapeutics, and they include, without limitation, medicaments; vitamins; mineral supplements; substances used for the treatment, prevention, diagnosis, cure or mitigation of a disease or illness; substances which affect the structure or function of the body; or pro-drugs, which become biologically active or more active after they have been placed in a physiological environment.

[0082] Specific examples of useful biologically active agents the above categories include: anti-neoplastics such as androgen inhibitors, alkylating agents, nitrogen mustard alkylating agents, nitrosourea alkylating agents, antimetabolites, purine analog antimetabolites, pyrimidine analog antimetabolites, hormonal antineoplastics, natural antineoplastics, antibiotic natural antineoplastics, carboplatin and cisplatin; nitrosourea

alkylating antineoplastic agents, such as carmustine (BCNU); antimetabolite antineoplastic agents, such as methotrexate; pyrimidine analog antineoplastic agents, such as fluorouracil (5-FU) and gemcitabine; hormonal antineoplastics, such as goserelin, leuprolide, and tamoxifen; natural antineoplastics, such as aldesleukin, interleukin-2, docetaxel, etoposide, interferon; paclitaxel, other taxane derivatives, and tretinoin (ATRA); antibiotic natural antineoplastics, such as bleomycin, dactinomycin, daunorubicin, doxorubicin, and mitomycin; vinca alkaloid natural antineoplastics, such as vinblastine and vincristine.

[0083] Other biologically active agents can include peptides, proteins, and other large molecules, such as interleukins 1 through 18, including mutants and analogues; interferons α, γ, and which may be useful for cartilage regeneration, hormone releasing hormone (LHRH) and analogues, gonadotropin releasing hormone transforming growth factor (TGF); fibroblast growth factor (FGF); tumor necrosis factor-α); nerve growth factor (NGF); growth hormone releasing factor (GHRF), epidermal growth factor (EGF), connective tissue activated osteogenic factors, fibroblast growth factor homologous factor (FGFHF); hepatocyte growth factor (HGF); insulin growth factor (IGF); invasion inhibiting factor-2 (IIF -2); bone morphogenetic proteins 1-7 (BMP 1-7); somatostatin; thymosin-α-γ-globulin; superoxide dismutase (SOD); and complement factors, and biologically active analogs, fragments, and derivatives of such factors, for example, growth factors.

[0084] In an embodiment, the additional biologically active agent can be another vaccine construct. Boursnell M. E. G. et al. describes the construction and characterization of a recombinant vaccinia virus vector (TA-HPV) expressing the tumor antigens E6 and E7 from HPV16 or HPV18 in "Construction and characterization of a recombinant vaccinia virus expressing human papillomavirus proteins for immunotherapy of cervical cancer." It was demonstrated that the recombinant virus, upon intraperitoneal administration to mice, has the capacity to prime a cytotoxic T lymphocyte (CTL) response against cells infected with the same virus vector or sensitized with a synthetic E7 peptide epitope. For example, the additional vaccine construct used in the inventive compositions and methods can include TA-HPV.

[0085] In accordance with one or more embodiments, the immunological compositions, including, for example, pNGVL4a-CRT/E7(detox) vaccine and TA-HPV vaccines are given by injection, e.g., i.m., i.p., i.v., subcutaneously, gene gun, etc. In some embodiments, the immunological compositions can be administered using electroporation.

[0086] It will be understood by those of ordinary skill in the art that the methods of the invention can be used in many variations of regimens, and should not be limited by any particular example.

[0087] In accordance with some embodiments, the treatment regimen used in the present invention comprises administration of a sufficient amount of the immunological compositions, including, for example, pNGVL4a-CRT/E7 (detox) plasmid vaccine systemically to the subject for a plurality of times, including, for example, administration of the vaccine at least two or more times, and in some embodiments, at least three times. There is generally an interval between administrations of at least 2 to 5 days, preferably about 3 days between administrations.

[0088] At sufficient time after the last administration of the immunological compositions to the subject, an adjuvant, such as a toll-like receptor 7 agonist, for example, is then administered locally or topically to the site of the HPV-related disease or infection. For example, for treatment of cervical cancer and related disease, the adjuvant is administered to the cervicovaginal tract of the subject.

[0089] Typically, the adjuvant will be administered on or about 5 to 10 days after the last administration of the immunological compositions to the subject. In a preferred embodiment, the adjuvant will be administered on or about 7 days after the last administration of the immunological compositions to the subject.

[0090] For other HPV-related disease, such as vulvar, vaginal, penile, oral, throat and anal cancers, for example, the adjuvant is administered topically to those affected areas.

[0091] In an embodiment, the adjuvant used in the treatment regimen is imiquimod.

EXAMPLES

[0092] Experimental mice and tumor cells. 6- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). IFN-γ receptor 1-deficient (*ifngr1*^{tm1Agt}) mice (IFNγR-/-) and CXCR3-deficient (B6.129P2-*Cxcr3*^{tm1Dgen}/J) mice (CXCR3-/-) have been previously described (Jackson Laboratories). Mice were housed in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

[0093] The tumor cell line used is TC-1. TC-1 cells were subjected to RapidMAP (Taconic Farms, Rensselaer, NY) testing, a panel of PCR tests for rodent viruses, most recently in May 2011 with negative results. In order to trace the tumor growth *in vivo*, TC-1 transfected with Luciferase (TC-1-Luc) was generated by lentivirus stable transfection as previously described (Human Gene Ther., 2007;18:575-88). Cells were cultured in RPMI1640 medium containing 10% FBS, 2mM L-glutamine, 10% sodium pyruvate, 10% non-essential amino acids, and 100 pg/ml streptomycin, in a humidified atmosphere of 5% CO_2/ 95% air at 37 °C.

[0094] Luciferase-expressing E7-specific CD8+ T cell and OT-1 T cell preparation and adoptive transfer in mice. E7-specific T cells were generated from splenocytes of E7 vaccinated mice and were stimulated with irradiated TC-1 cells and 10 IU interleukin-2 (IL-2) weekly. In order to trace the E7-specific CD8+ T cells *in vivo*, we have previously generated E7-specific cytotoxic T cells expressing luciferase (E7-Luc T cell). OT-1 T cells were generated from OT-1 Rag-/- TCR transgenic mice from our lab as previously mentioned (Gene Ther., 2010;17:1453-64). T cell adoptive transfer was performed by injecting $5x10^5$ T cells in 200μL PBS through the tail veins of mice.

[0095] DNA vaccine and drug treatments. The pNGVL4a-CRT/E7 (detox) plasmid was prepared by the NIH Rapid Access to Interventional Development (RAID) program. Imiquimod (Aldara) 5% cream was purchased from Taro Pharmaceutical Industries, Ltd (Haifa Bay, Israel). Mice were injected in the tibialis muscle of the shaved hind leg with a 28G syringe containing 40 μg of DNA plasmid diluted in a total volume of 40 μL of PBS. Each leg was injected with 20 μg DNA vaccine. After the mice were under anesthesia with ketamine (75 mg/kg)/ xylazine (20 mg/kg), 0.4 mg of imiquimod was applied in the vagina using a micropipette tip.

[0096] *In vivo* luciferase-based bioluminescence imaging. Luciferin (Sigma) was used to test for firefly luciferase activity *in vivo*. Mice were injected with the substrate luciferin 40 mg/kg intraperitoneally and sedated by inhaling isoflurane USP (Baxter International, Inc). Bioluminescence of the mice was detected via the IVIS Imaging System 200 Series. The region of interest from displayed images was designated and quantified as total photon counts using Living Image 2.50 software (Xenogen).

[0097] Orthotopic cervical cancer model and treatment. Mice (5 per group) were challenged intravaginally with 2 x 10^4 TC-1-Luc cells/mice using methods previously described (Human Gene Ther., 2011;22:809-19). Briefly, four days before the tumor implantation, female mice were diestrus synchronized by injection of medroxyroprogesterone (Greenstone LLC, NJ) s.c. (3 mg/mouse), and all vaginal procedures performed upon isoflurane anesthesia. Mice were administered 4% nonoxynol-9 (N9, Igepal, Sigma) intravaginally one day before tumor challenge. On day one, the genital tracts were washed with PBS before injecting 2 x 10^4 TC-1-Luc cells into the vaginal tract. Tumor growth was confirmed by IVIS 2000 system on day 7 before immunization began. Mice were vaccinated with 40 μg of CRT/E7 DNA plasmid via intramuscular injection 3 times at 3 day intervals. 0.4 mg of imiquimod 5% cream was deposited in the vagina on day 13 after the last boost vaccination and was subsequently applied every seven days. Mice were euthanized when they demonstrated stress or when they lost more than 20% of their body weight, based on animal care regulations.

[0098] Analysis of the immune cells in vaginal and tumor tissues. The genital tracts and tumor tissue of mice were dissected. Tissue samples were cut into small pieces and digested with 0.05 mg/ml collagenase I, 0.05 mg/ml collagenase IV, 0.025 mg/ml hyaluronidase IV, 0.25 mg/ml DNase I, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C for 60 minutes as previously described (Cancer Immunolo. Immunother., CII. 2013;62:171-82; Int. J. Cancer, 2011;128:2105-13). The tissue digest was then filtered through a 70 μm nylon filter mesh to remove undigested tissue fragments.

[0099] Flow cytometry analysis and intracellular cytokine staining to detect IFN-γ secretion by E7-specific $CD8^+$ T cells in the tumor microenvironment. H2-$2D^b$ tetramers labeled with phycoerythrin (PE) and complexed to the HPV-16 E7 (RAHYNIVTF) peptide were gifts from the NIH. Single-cell suspended splenocytes, genital lymph nodes, and cervicovaginal cells from immunized and control mice were incubated in FACS buffer (0.5% BSA, 2 mM EDTA, 0.1% NaN_3 in PBS). FITC-labeled anti-CD8a antibodies (BD 53-6.7)

and PE-labeled tetramers were added and incubated at 4 °C for 30 minutes. Samples were also stained with 7-AAD to exclude dead cells (BD). For analysis of the expression of integrin and chemokine receptors, CD8+ T cells were co-stained with anti-mouse CD103 APC-mAb (eBioscience 2E7), anti-mouse CD49a Alexa Fluor 647 (BD Ha3118) mAb, and CXCR3/CD183 APC-mAB (BD CXCR3-173). Cells were incubated with Fc blocker anti-CD16/CD32 (eBioscience) at 4 °C for 5 minutes.

[00100] Cell surface marker staining for anti-mouse PE CD8 (BD RPA-T8) and intracellular cytokine staining for anti-mouse FITC IFN-γ (BD XMG1.2) as well as FACScan analysis were performed in the same conditions as those previously described (PloS one. 2013;8:e56912). Lymphocytes extracted from tumor fragments were collected and incubated with 1 μg/ml of E7 peptide (RAHYNIVTF) (SEQ ID NO: 1) as previously described (J. Clin. Invest., 2003;112:109-17) in 24-well plates for 8 hours. The number of IFN-γ-secreting CD8+ T cells was analyzed by FACScan cytometry. All analyses were performed with Flowjo 10.1.

[00101] Quantitative real-time PCR. mRNA from vaginal tissue was extracted using Trizol after homogenization. First strain cDNA was synthesized by reverse transcription, according to the manufacturer's protocol (BioRad). Then, the first strain cDNA was used for quantitative real-time PCR using IQ SYBR Green (Invitrogen) on the MyiQ real-time detection system (BioRad) following the manufacturer's protocol. The forward and reverse primers for β-actin sense-ACTGGGACGACATGGAGAAG, (SEQ ID NO: 2) antisense-GGGGTGTTGAAGGTCTCAAA, (SEQ ID NO: 3) TLR-7 sense-CCACAGGCTCACCCATACTTC, (SEQ ID NO: 4) antisense-GGGATGTCCTAGCTGGTGACA, (SEQ ID NO: 5) CXCR-9 sense-CAAATCCCTCAAAGACCTCAAAC , (SEQ ID NO: 6) antisense-GATCTCCGTTCTTCAGTGTAGC, (SEQ ID NO: 7) CXCR-10 sense-TCATCCCTGCGAGCCTAT , (SEQ ID NO: 8) antisense-CTTGATGGTCTTAGATTCCGGAT, (SEQ ID NO: 9) and IFN-γ sense-ACAATGAACGCTACACACTGCAT, (SEQ ID NO: 10) antisense-TGGCAGTAACAGCCAGAAACA, (SEQ ID NO: 11). Gene expression levels were normalized to β-Actin housekeeping gene, and data were represented as fold differences by the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct_{target\ gene} - Ct_{\beta\text{-}Actin}$ and $\Delta\Delta Ct = \Delta Ct_{induced} - \Delta Ct_{reference}$, as previous mentioned (J Immunol. 2012;189:2985-94).

[0100] Statistical analysis. All data are expressed as mean ± S.E. where indicated. Comparisons between individual data points for intracellular cytokine staining with flow cytometric analysis and tumor treatment were made using Student's t-test. In the tumor treatment experiments, the principal outcome of interest was duration until mice were sacrificed for ethical reasons (in stress, body weight lost is greater than 20%). The event time distributions for different mice were compared using the Kaplan–Meier method and the log-rank statistic by Prism 6 software. All p-values <0.05 were considered significant.

EXAMPLE 1

[0101] Imiquimod and N9 deposition in the vagina both increase the population of E7-specific $CD8^+$ T cells locally. First, to examine the impact of inflammation in the genital tract, we examined the systemic and local effects of imiquimod treatment on the cervicovaginal tract after vaccination. Mice were vaccinated with 40 μg pNGVL4a-CRT/E7 (detox) plasmid vaccine via intramuscular injection in the hind legs on days 1, 4 and 8. Imiquimod, or as a control PBS, was then applied to the vaginal tract of mice on day 15. Peripheral blood and vaginal tissue were collected on day 11. Mice treated with either imiquimod or PBS generated the similar levels of E7-specific $CD8^+$ T cells in the circulation (Figures 1A and 1B). However, we observed that the genital tract of mice treated intravaginally with imiquimod displayed a 10-fold increase in total $CD8^+$ T cells compared with mice treated with PBS (Figure 1C). Furthermore, the number of E7-specific $CD8^+$ T cells in the vaginal tissue was also dramatically increased (8.2 fold) in mice treated with imiquimod compared to those treated with PBS, but suggests that the enrichment of activated T cells is not antigen-specific (Figure 1D and 1E). This suggests that local imiquimod application enhances local replication of the CD8+ T cells and/or recruits them to the site of treatment.

[0102] N9 is used as a topical spermicide and has previously been shown to trigger a local inflammatory response after vaginal application. Interestingly, we also observe an increase in the number of E7-specific CD8+ T cells in the cervicovaginal tract of mice after i.m. vaccination with pNGVL4a-CRT-E7 (detox) and subsequent intravaginal deposition of N9 compared to mice administered only PBS as a control (Figure 9). This suggests that local application of imiquimod (or N9) enhances local replication of the CD8+ T cells and/or recruits them to the site of treatment.

EXAMPLE 2

[0103] Imiquimod application attracts luciferase-expressing E7-specific CD8+ T cells to the cervicovaginal tract. In order to determine if application of imiquimod attracts luciferase-expressing E7-specific $CD8^+$ T cells in the cervicovaginal tracts of treated mice, we applied imiquimod or PBS to the cervicovaginal tracts of mice and then adoptively transferred the E7-Luc $CD8^+$ T cells (5×10^5/mouse) via tail vein. After 48 hours, the entire genital tract was excised in order to measure bioluminescence intensity and thereby detect presence of the luciferase-expressing E7-specific $CD8^+$ T cells (Figures 7A-7B). Figure 7B shows that a significantly higher number of E7-Luc T cells were present in the cervicovaginal tracts of mice treated with imiquimod compared to those treated with PBS. This data indicates that the application of imiquimod attracts antigen-specific $CD8^+$ T cells in to the genital tract.

EXAMPLE 3

[0104] Systemic CRT/E7 DNA vaccination combined with local imiquimod application generated potent antitumor effects in the orthotropic cervical cancer model. Next, the combination strategy of systemic vaccination with local imiquimod application was tested to determine if it could improve the treatment of an orthotropic genital mucosal tumor model, luciferase-expressing TC-1 tumor cells growing in the vaginal wall. Mice bearing vaginal TC-1-luc tumors one week after inoculation were divided into four groups, naïve, vaccination only (VAC), imiquimod only (IMQ), and vaccination plus imiquimod (VAC+IMQ) and their treatment schedule is illustrated in Figure 2A.

[0105] As shown in Figure 2B, the bioluminescence signals, indicating vaginal tumor load, in all groups of mice were not significantly different on day 7 and increased to a similar level by day 14. Subsequently, the signal dramatically decreased in the VAC+IMQ group by day 21. In contrast, the signals in the mice of the remaining groups continued to steadily increase, although the VAC only and IMQ only signals were about 10-fold lower than the control mice. Figure 2C shows that as early as day 21, the tumors of mice treated with DNA vaccine and imiquimod were significantly smaller than those of the other treatment groups. These results correlated with the survival rates of the groups of mice. Although mice in the VAC group and IMQ group showed improved survival compared to the control mice, mice in

the VAC+IMQ group had significantly prolonged survival compared to both the VAC group ($p<0.01$) and the IMQ group ($p<0.01$) (Figure 8A). All the mice in VAC+IMQ group survived until day 70 without any sign of tumor recurrence. Furthermore, mice treated with DNA vaccination and imiquimod had significantly higher numbers of total CD8+ T cells and E7-specific CD8+ T cells in the tumor loci compared to those in the other treatment groups (Figures 8B and 8C). Here, the fold increase in E7-specific CD8+ T cells was greater than total CD8+ T cell accumulation, suggesting preferential accumulation of the activated CD8+ T cells in the tumor because of the presence of cognate antigen. Taken together, the data shown in Figure 2, and Figures 7 and 8, show that intravaginal application of imiquimod leads to the accumulation in the cervicovaginal tumor of systemic E7-specific $CD8^+$ T cells generated by CRT/E7 DNA plasmid vaccination, resulting in the most potent antitumor effect against E7-expressing orthotopic tumors in the cervicovaginal tract.

EXAMPLE 4

[0106] Activation of antigen-specific CD8+ T cells in the cervicovaginal tract leads to accumulation of other antigen-specific CD8+ T cells in the cervicovaginal tract. It was further explored whether the activation of antigen-specific CD8+ T cells in the cervicovaginal tract would create a suitable environment to attract other antigen-specific CD8+ T cells. Naïve C57BL/6 mice and tumor-free mice previously challenged with TC-1-Luc cells and effectively treated with CRT/E7 DNA vaccine and imiquimod (antigen-experienced mice) were used to further characterize the attraction of other antigen-specific CD8+ T cells to the cervicovaginal tract. As outlined in Figure 3A, mice were administered with E7 peptide (RAHYNIVTF) (SEQ ID NO: 1) in the cervicovaginal tract one day before adoptive transfer of OVA-specific CD8+ T cells (OT-1). 5×10^5 OT-1 T cells were labeled with CFSE before injection into mice via the tail vein. Two days after adoptive transfer, splenocytes and vaginal tissue were collected and CFSE-labeled OT-1 T cells were quantified by flow cytometry analysis. We observed similar numbers of OT-1 T cells in the circulation after adoptive transfer in all treatment groups (Figures 3B and 3C). Importantly, there were a significantly greater number of OT-1 T cells that accumulated in the vaginal tracts in antigen-experienced mice that received E7 peptide administration but not in antigen-experienced mice that did not receive E7 peptide administration (Figures 3B and 3D). These results

suggest that local inflammation induced by the activation of antigen-specific CD8+ T cells can recruit activated CD8+ T cells regardless of antigen specificity to the vaginal tissue.

EXAMPLE 5

[0107] Imiquimod induced local expression of CXCL9 and CXCL10 leading to CXCR3+ cytotoxic T cell accumulation in the cervicovaginal tract. Previous studies have shown that imiquimod application on the cervix elevated the expression of the chemokines CXCL9 and CXCL10 in the local tissue in murine and non-human primate models. In order to characterize the expression of CXCL9 and CXCL10 mRNA, qRT-PCR was performed using mRNA derived from the cervicovaginal tracts following local imiquimod treatment. Treatment with PBS was used as a control for comparison. As shown in Figure 4A, we found that the CXCL9 and CXCL10 mRNA expression was significantly higher in imiquimod treated vaginal tissue compared to PBS treated tissue. Because CXCR3, the receptor for both CXCL9 and CXCL10, is expressed on T cells and functions to induce T cell migration to tissues displaying danger signals, we also examined the mRNA expression level of CXCR3 using qRT-PCR on $CD8^+$ T cells isolated from the cervicovaginal tract. Treatment with imiquimod induced a significantly increased number of CXCR3-expressing $CD8^+$ T cells recovered from the genital tract as compared to treatment with PBS (Figure 4B). Additionally, we compared the expression of surface markers associated with T cell migration to lesions, CD103, $\alpha 4\beta 7$, and CD49a, on genital tract T cells of control mice versus those treated with imiquimod. Figure 4B shows that in imiquimod treated mice there was a significantly higher proportion of CD49a+ T cells in the cervicovaginal tract compared to mice treated with PBS.

EXAMPLE 6

[0108] IFNγ signaling is important for both CXCL9/10 expression and accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract following imiquimod treatment.

[0109] IFNγ signaling induces CXCL9/10 expression. In the present study, we found that IFNγ expression was upregulated after local imiquimod treatment (Figure 4A). This implies that IFNγ may be a key cytokine governing CXCL9/10 upregulation and the attraction of the activated CD8+ T cell immune response after imiquimod application. In order to determine if the IFNγ signal pathway is important for the upregulated expression of CXCL9/10 in the cervicovaginal tract, we compared the genital tracts of IFNγ receptor knockout mice (IFNγR -/-) with wild type mice (Wt) following imiquimod treatment for their levels of TLR7, IFNγ, CXCL9, and CXCL10 mRNA. We found that the expression levels of IFNγ, CXCL9, and CXCL10 were significantly reduced in IFNγR -/- mice compared to Wt mice (Figure 5A). In comparison, the expression level of TLR7 was not significantly different between IFNγR -/- mice and Wt mice.

[0110] In order to demonstrate that IFNγ is important for the accumulation of antigen-specific CD8+ T cells in the cervicovaginal tract following imiquimod treatment, we compared the number of E7-specific CD8+ T cells in the cervicovaginal tracts of Wt and IFNγR-/- mice following DNA vaccination and local imiquimod application. As shown in Figure 5B, while the numbers of E7-specific CD8+ T cells in PBMCs were similar between Wt and IFNγR -/- mice, there were significantly fewer CD8+ T cells (Figure 5C) and E7-specific CD8+ T cells in the cervicovaginal areas of IFNγR-/- mice compared to Wt mice (Figure 5D and E). Taken together, these data suggest that the IFNγ signal pathway plays an important role in triggering CXCL9/10 chemokine expression and the accumulation of E7-specific CD8+ T cells in the cervicovaginal tract following imiquimod treatment.

EXAMPLE 7

[0111] CXCR3 is crucial for antigen-specific CD8+ T cell accumulation in imiquimod-treated cervicovaginal tract. In Figure 4B, we showed that CXCR3+ CD8+ T cells accumulated in imiquimod-treated cervicovaginal tissue. To test the importance of CXCR3 expression on the CD8+ T cells for accumulation in the genital tract upon imiquimod treatment, we compared the number of CD8+ T cells in Wt mice and CXCR3-/- mice. All

mice were vaccinated systemically with CRT/E7 DNA followed by treatment with imiquimod in the cervicovaginal tract. As shown in Figure 6, we found that in the cervicovaginal tract, the total number of CD8+ T cells and E7-specific CD8+ T cells were significantly reduced in CXCR3-/- mice compared to Wt mice. Taken together, these data suggest that the expression of CXCR3 on CD8+ T cells is crucial for the presence of antigen-experienced cytotoxic T cells in CXCL9/10-expressing tissue following local imiquimod application.

[0112] The present invention provides 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.

[0113] 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.

[0114] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by

applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Claims:

1. A composition comprising a therapeutically effective amount of a vaccine composition comprising HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered systemically to the subject and wherein the adjuvant is administered to the subject to the site of the HPV-related disease or infection in the subject.

2. The composition of claim 1, wherein the vaccine is a fusion protein vaccine comprising HPV16, HPV18 or HPV6 E6E7L2 or L2E7.

3. The composition of claim 1, wherein the vaccine composition is administered to the subject before administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

4. The composition of claim 1, wherein the vaccine composition is administered to the subject concurrently with administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

5. The composition of claim 1, wherein the vaccine composition and adjuvant composition are repeatedly administered to the subject at specific time intervals.

6. The composition of claim 5, wherein the time intervals are 3 days to 60 days apart.

7. The composition of any of claims 1 to 6, wherein the adjuvant is a toll-like receptor (TLR) ligand or a combination of two or more ligands.

8. The composition of claim 7, wherein the TLR ligand is a TLR agonist.

9. The composition of claim 7, wherein the TLR agonist is selected from the group consisting of imiquimod, monophosphoryl lipid A (MPL), bacillus Calmette–Guérin (BCG), Polyinosinic-polycytidylic acid (poly(I:C) and resiquimod.

10. The composition of any of claims 1 to 6, wherein the adjuvant is Nonoxynol-9.

11. A composition comprising a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered systemically to the subject and wherein the adjuvant is administered to the subject to the site of the HPV-related disease or infection in the subject.

12. The composition of claim 11, wherein the vaccine composition comprises a pNGVL4a-CRT/E7(detox) plasmid.

13. The composition of claim 11, wherein the vaccine composition comprises a pNGLV4a-CRTE6E7L2 plasmid.

14. The composition of claim 11, wherein the vaccine composition comprises a pNGLV4a-sigE7(detox)HSP70 plasmid.

15. The composition of claim 11, wherein the vaccine composition comprises a recombinant vaccinia virus expressing HPV16 and HPV18 E6 and E7.

16. The composition of claim 11, wherein the vaccine composition comprises a prime-boost combination of a fusion protein HPV6, HPV16 and/or HPV18 E6 and/or E7 and a polynucleotide expressing HPV16 and/or HPV18 E6 and/or E7.

17. The composition of claim 11, wherein the vaccine composition comprises a prime-boost combination of polynucleotide expressing HPV16 and/or HPV18 E6 and/or E7 and a recombinant vaccinia virus expressing HPV16 and HPV18 E6 and E7.

18. The composition of claim 11, wherein the vaccine composition comprises a prime-boost combination of polynucleotide expressing HPV16 and/or HPV18 E6 and/or E7 and a fusion protein HPV6, HPV16 and/or HPV18 E6 and/or E7.

19. The composition of claim 11, wherein the vaccine composition is administered to the subject before administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

20. The composition of claim 11, wherein the vaccine composition is administered to the subject concurrently with administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

21. The composition of claim 11, wherein the vaccine composition and adjuvant composition are repeatedly administered to the subject at specific time intervals.

22. The composition of claim 21, wherein the time intervals are 10 days to 60 days apart.

23. The composition of any of claims 11 to 22, wherein the adjuvant is a toll-like receptor (TLR) ligand or a combination of two or more ligands.

24. The composition of claim 23, wherein the TLR ligand is a TLR agonist.

25. The composition of claim 24, wherein the TLR agonist is selected from the group consisting of imiquimod, monophosphoryl lipid A (MPL), bacillus Calmette–Guérin (BCG), Polyinosinic-polycytidylic acid (poly(I:C) and resiquimod.

26. The composition of any of claims 11 to 22, wherein the adjuvant is Nonoxynol-9.

27. The composition of any of claims 1 to 6, or 11 to 22, wherein the HPV-related disease or infection is selected from the group consisting of HPV infection at any body site, skin warts, genital warts, intraepithelial neoplasia, ASCUS, AGUS, HPV+ associated cancers such as cancer cervical, vulvar, vaginal, penile, oral, throat and anal cancers, also including non-melanoma skin cancer.

28. The composition of any of claims 1 to 6, or 11 to 22, wherein the at least two administrations of the vaccine composition to the subject is separated in time by 1 to 100 days, and wherein the administration of a sufficient amount of adjuvant to the site of the HPV-related disease or infection in the subject is at least 5 to 10 days after the last administration of the vaccine composition.

29. The composition of claim 28, wherein the administration of a sufficient amount of the immunological composition systemically to the subject is three times.

30. The composition of any of claims 11 to 22, wherein the amount of vaccine composition administered is between 100 pg to 4000 μg.

31. The composition of any of claims 1 to 6, or 11 to 22, wherein the adjuvant is imiquimod, and the amount of imiquimod administered is between 0.1 to 100 mg.

32. The composition of any of claims 11 to 22, wherein the immunological composition is administered by injection, gene gun, or electroporation.

33. The composition of any of claims 1 to 6, or 11 to 22, further comprising administration of at least one additional biologically active agent.

34. The composition of claim 14, wherein the adjuvant is imiquimod, and wherein the at least two administrations of the vaccine composition to the subject is separated in time by 28 days, and wherein the administration of a sufficient amount of adjuvant to the site of the HPV-related disease or infection in the subject is at least 5 to 10 days after the last administration of the vaccine composition.

35. A composition comprising a therapeutically effective amount of a vaccine composition comprising a polynucleotide encoding HPV associated proteins selected from the group consisting of E1, E2, E4, E5, E6, E7, L1 and L2, and an effective amount of a composition comprising an adjuvant, for use in generating an immune response against human papillomavirus (HPV)-associated disease in a subject, wherein the vaccine composition is administered to the subject at the site of the HPV-related disease or infection in the subject and wherein the adjuvant is administered to the subject at the site of the HPV-related disease or infection in the subject.

36. The composition of claim 35, wherein the vaccine composition comprises a pNGVL4a-CRT/E7(detox) plasmid.

37. The composition of claim 35, wherein the vaccine composition comprises a pNGLV4a-CRTE6E7L2 plasmid.

38. The composition of claim 35, wherein the vaccine composition comprises a pNGLV4a-sigE7(detox)HSP70 plasmid.

39. The composition of claim 35, wherein the vaccine composition is administered to the subject before administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

40. The composition of claim 35, wherein the vaccine composition is administered to the subject concurrently with administration of the adjuvant to the site of the HPV-related disease or infection in the subject.

41. The composition of claim 35, wherein the vaccine composition and adjuvant composition are repeatedly administered to the subject at specific time intervals.

42. The composition of claim 35, wherein the time intervals are 10 days to 60 days apart.

43. The composition of claim 35, wherein the adjuvant is a toll-like receptor (TLR) ligand or a combination of two or more ligands.

44. The composition of claim 43, wherein the TLR ligand is a TLR agonist.

45. The composition of claim 44, wherein the TLR agonist is selected from the group consisting of imiquimod, monophosphoryl lipid A (MPL), bacillus Calmette–Guérin (BCG), Polyinosinic-polycytidylic acid (poly(I:C) and resiquimod.

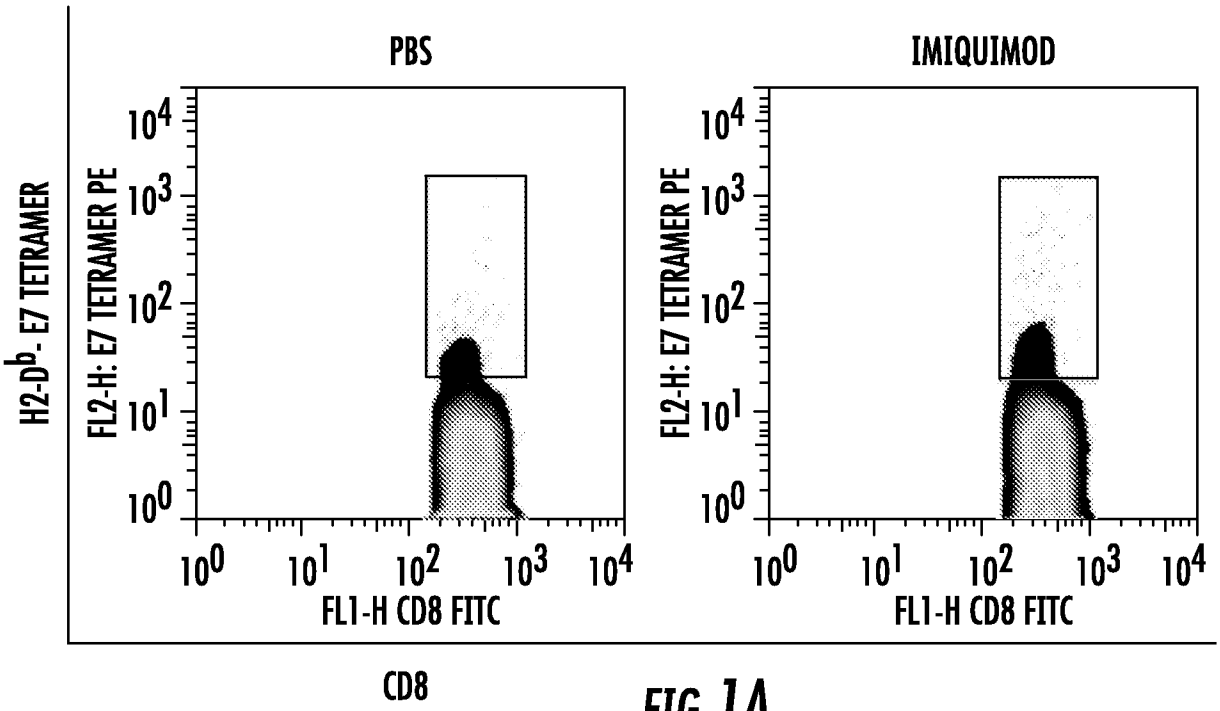

PBS
IMIQUIMOD
H2-D^b- E7 TETRAMER
FL2-H: E7 TETRAMER PE
FL1-H CD8 FITC
CD8

FIG. 1A

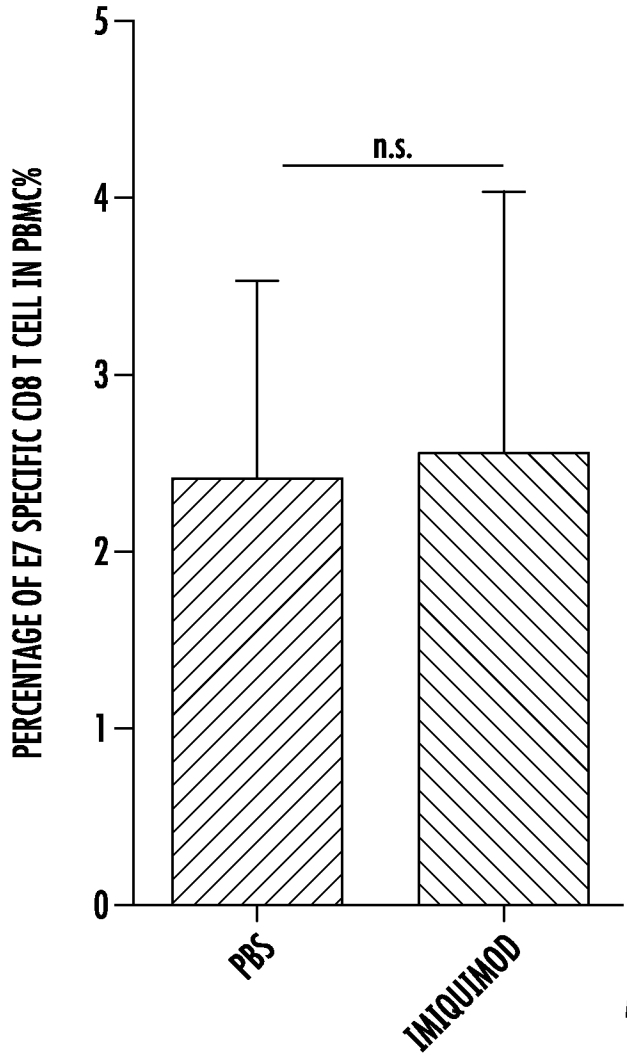

PERCENTAGE OF E7 SPECIFIC CD8 T CELL IN PBMC%
n.s.
PBS
IMIQUIMOD

FIG. 1B

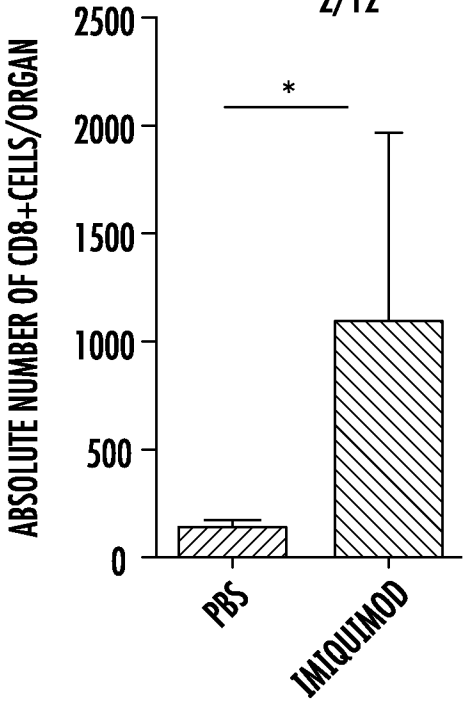

ABSOLUTE NUMBER OF CD8+CELLS/ORGAN
2500
2000
1500
1000
500
0
*
PBS
IMIQUIMOD

FIG. 1C

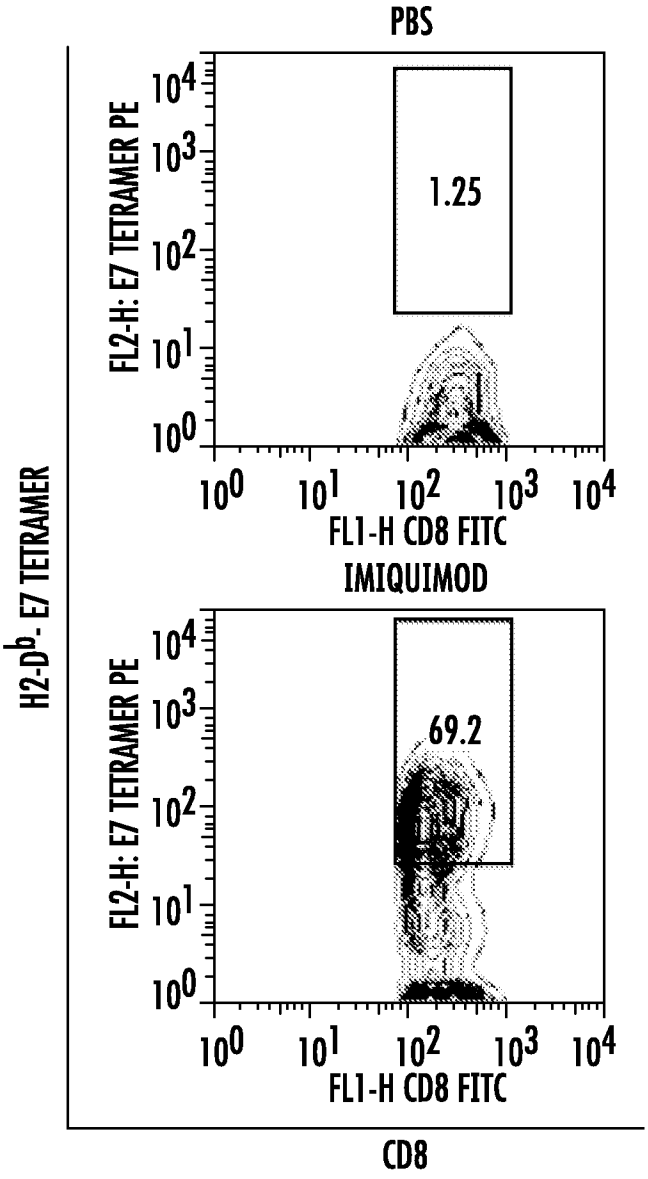

PBS
IMIQUIMOD
H2-D^b- E7 TETRAMER
FL2-H: E7 TETRAMER PE
FL1-H CD8 FITC
1.25
69.2
CD8

FIG. 1D

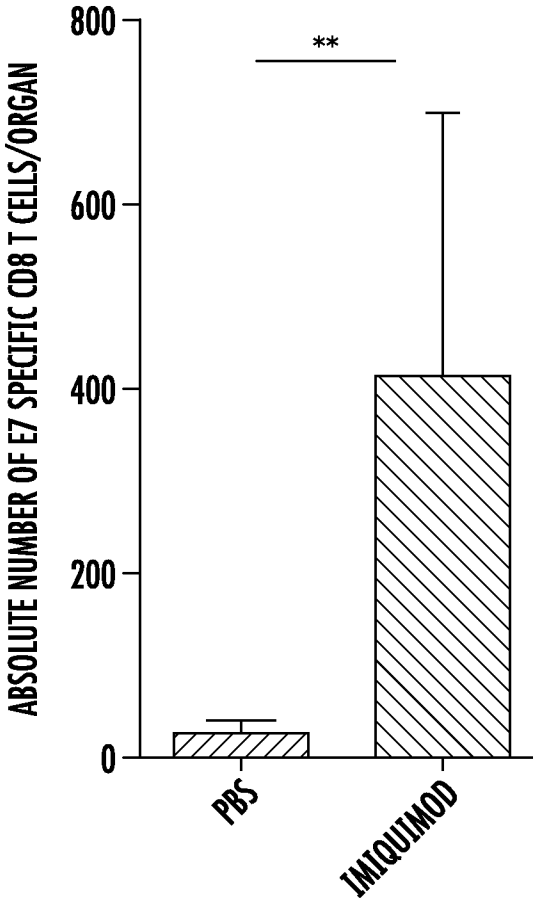

800
600
400
200
0
**
ABSOLUTE NUMBER OF E7 SPECIFIC CD8 T CELLS/ORGAN
PBS
IMIQUIMOD

FIG. 1E

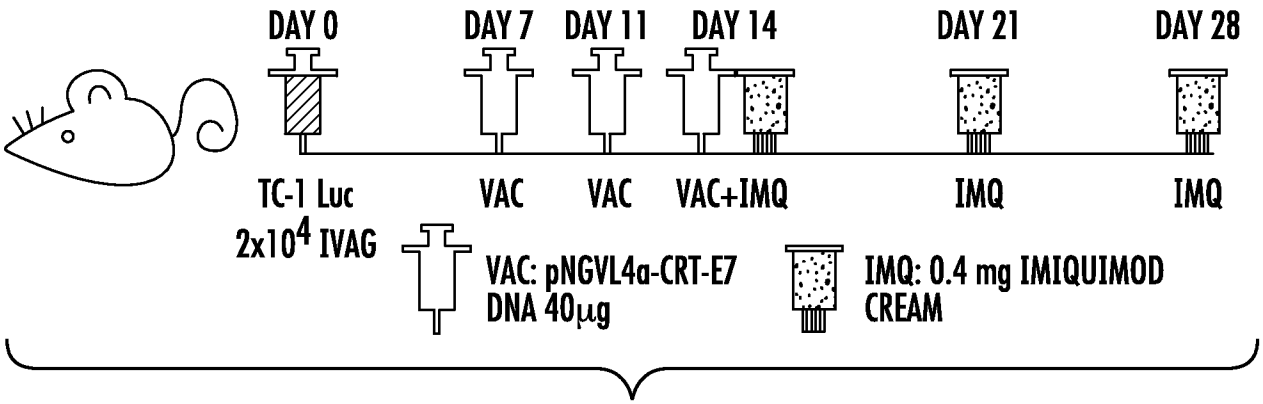

DAY 0
DAY 7
DAY 11
DAY 14
DAY 21
DAY 28
TC-1 Luc
2x10^4 IVAG
VAC
VAC
VAC+IMQ
IMQ
IMQ
VAC: pNGVL4a-CRT-E7
DNA 40μg
IMQ: 0.4 mg IMIQUIMOD
CREAM

FIG. 2A

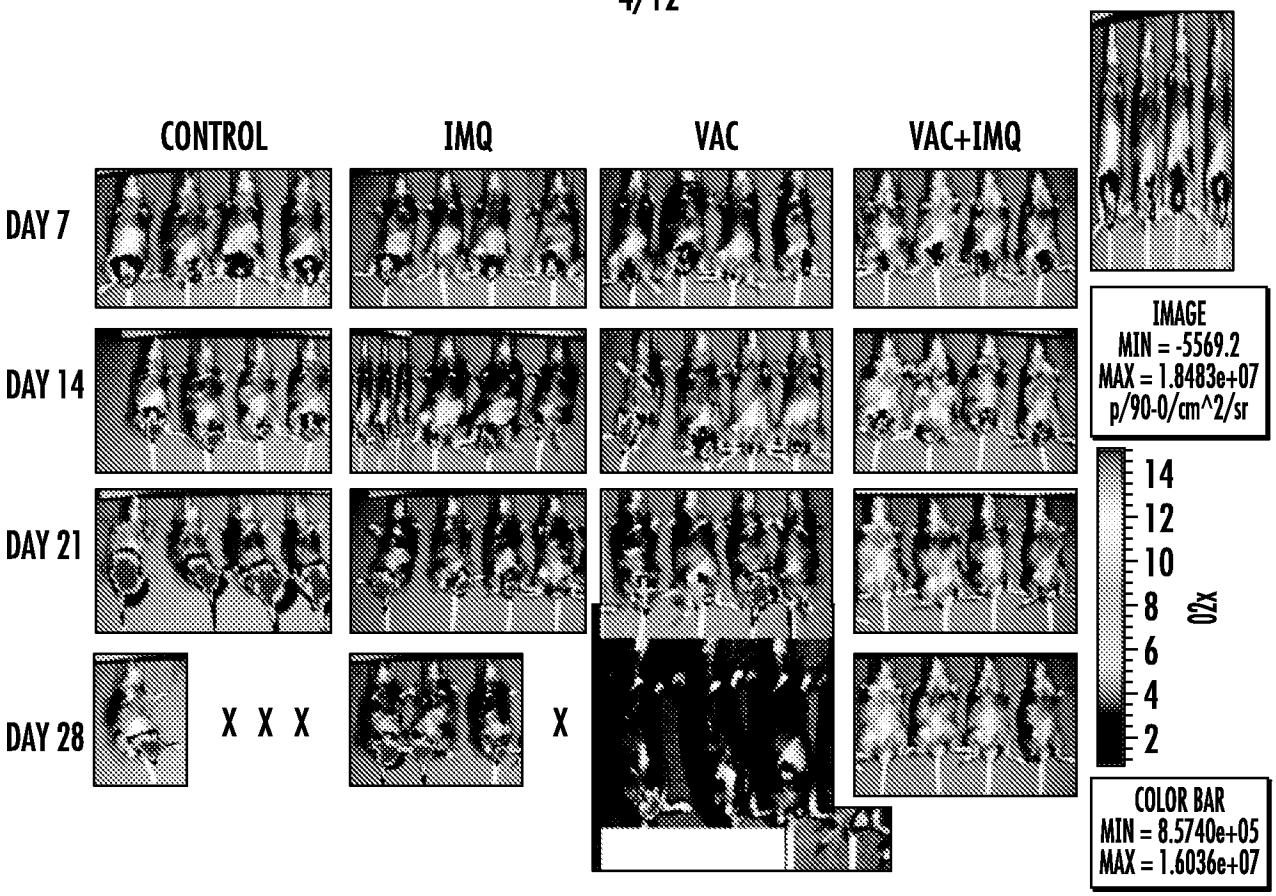

CONTROL
IMQ
VAC
VAC+IMQ
DAY 7
DAY 14
DAY 21
DAY 28
X X X
X
IMAGE
MIN = -5569.2
MAX = 1.8483e+07
p/90-0/cm^2/sr
14
12
10
8
6
4
2
02x
COLOR BAR
MIN = 8.5740e+05
MAX = 1.6036e+07

FIG. 2B

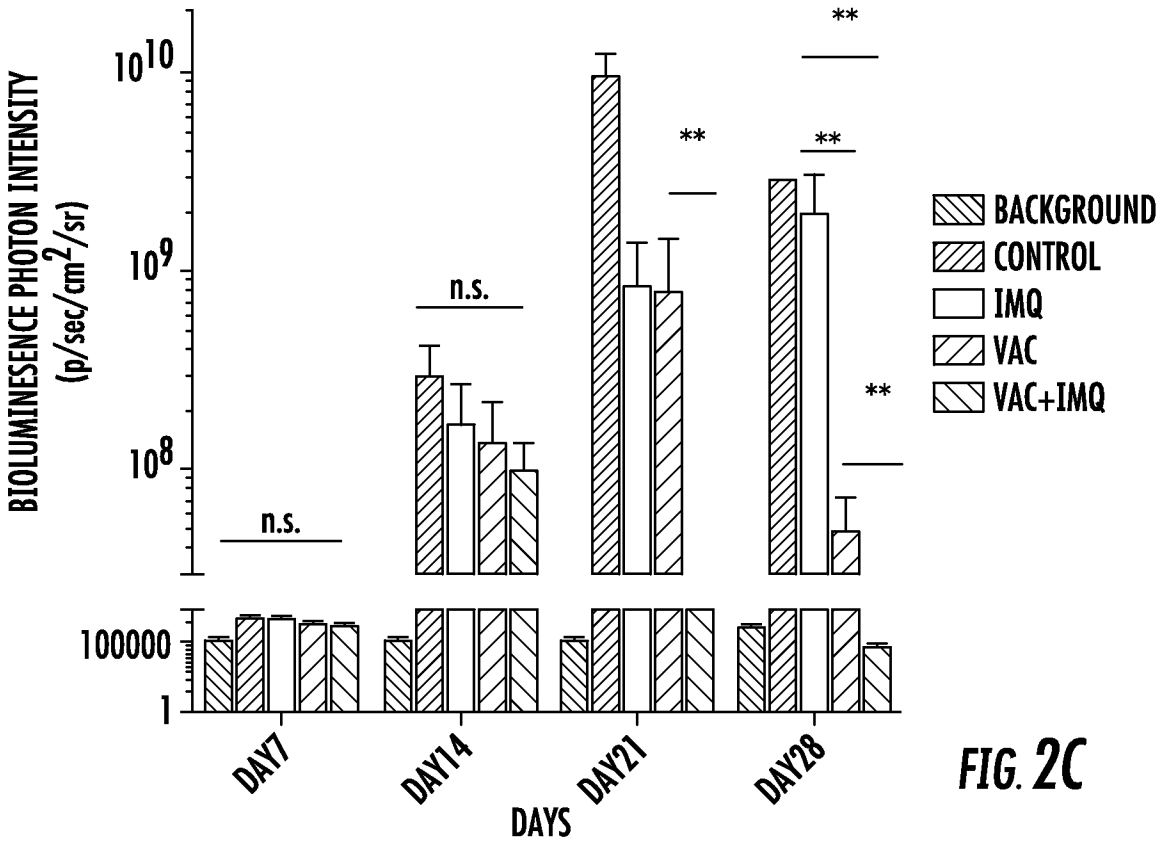

BIOLUMINESCENCE PHOTON INTENSITY
(p/sec/cm2/sr)
10^10
10^9
10^8
100000
1
n.s.
n.s.
**
**
**
**
BACKGROUND
CONTROL
IMQ
VAC
VAC+IMQ
DAY7
DAY14
DAY21
DAY28
DAYS

FIG. 2C

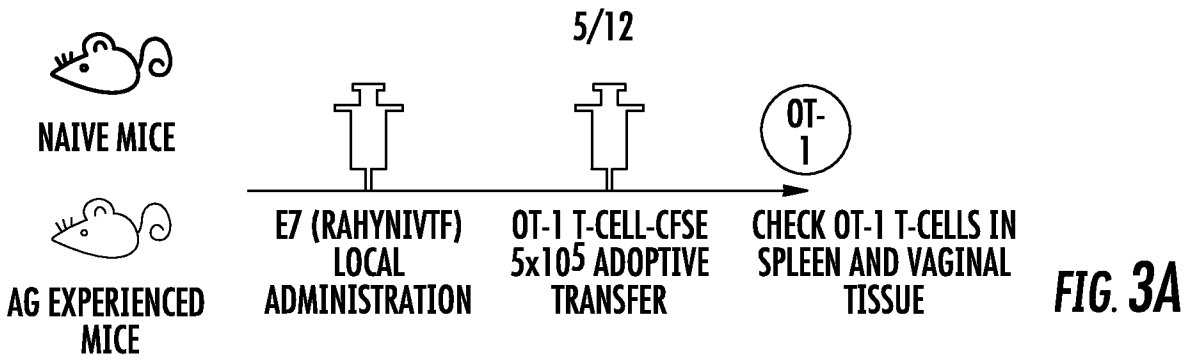

5/12
NAIVE MICE
AG EXPERIENCED MICE
E7 (RAHYNIVTF) LOCAL ADMINISTRATION
OT-1 T-CELL-CFSE 5x10^5 ADOPTIVE TRANSFER
OT-1
CHECK OT-1 T-CELLS IN SPLEEN AND VAGINAL TISSUE

FIG. 3A

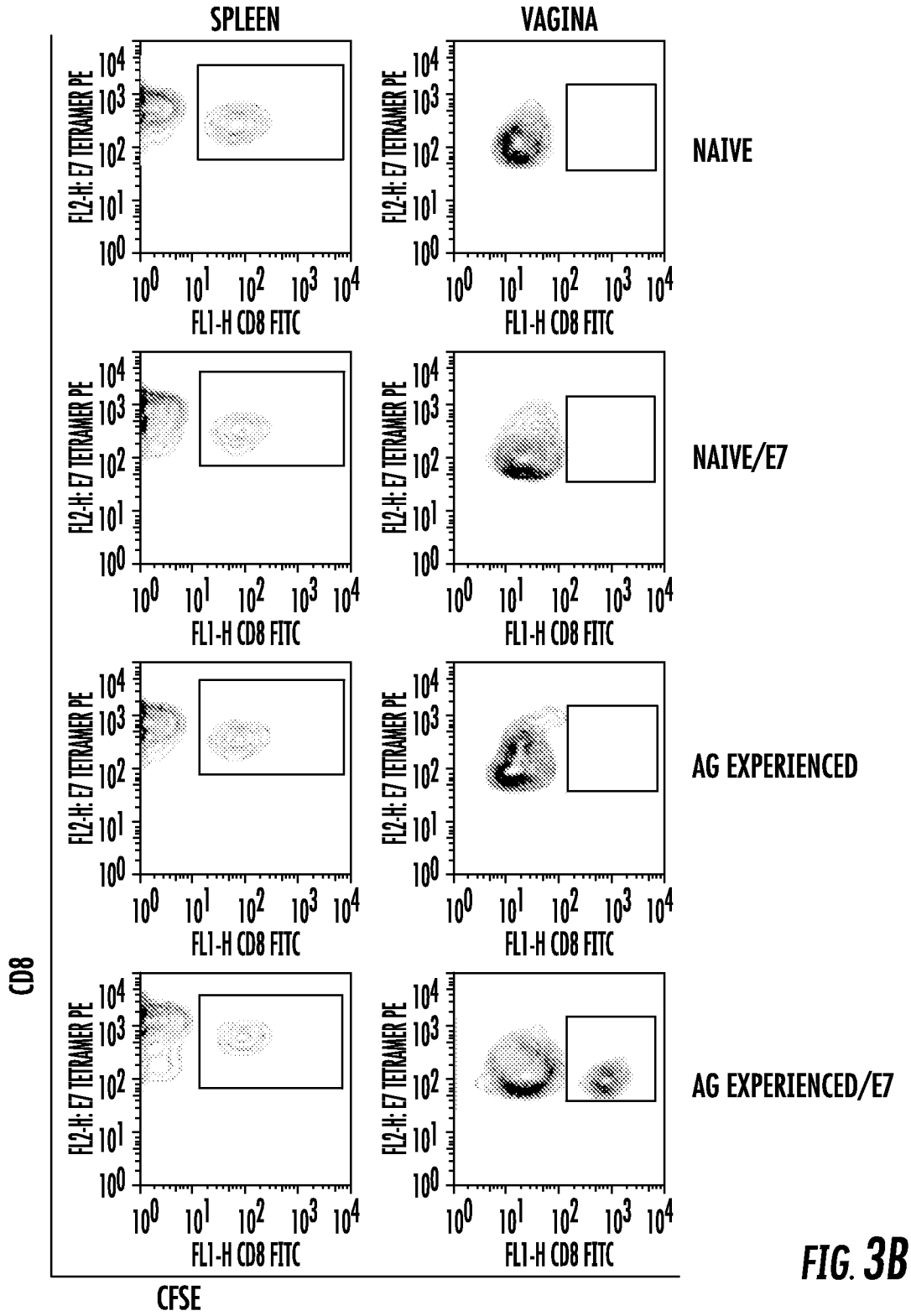

SPLEEN
VAGINA
NAIVE
NAIVE/E7
AG EXPERIENCED
AG EXPERIENCED/E7
FL2-H: E7 TETRAMER PE
FL1-H CD8 FITC
CD8
CFSE

FIG. 3B

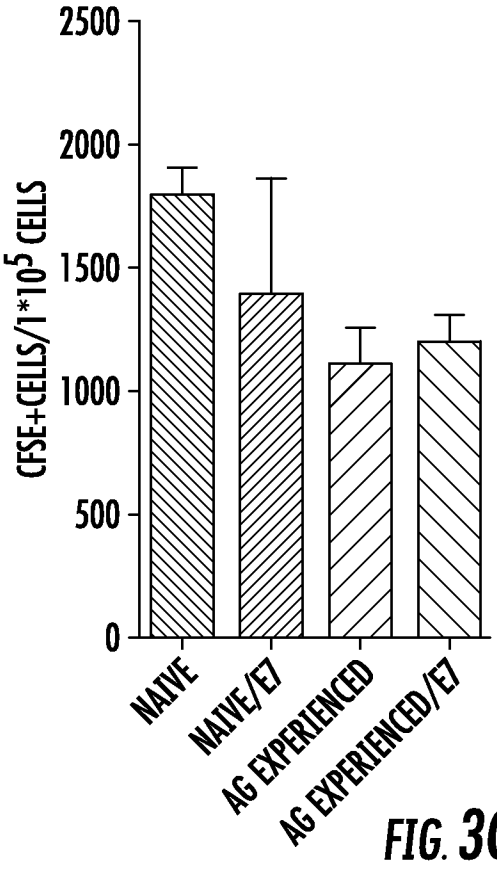


FIG. 3C

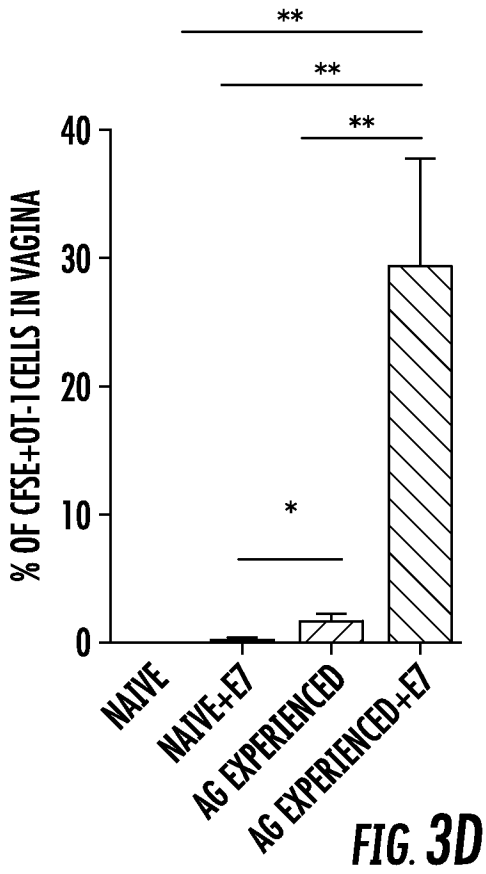


FIG. 3D

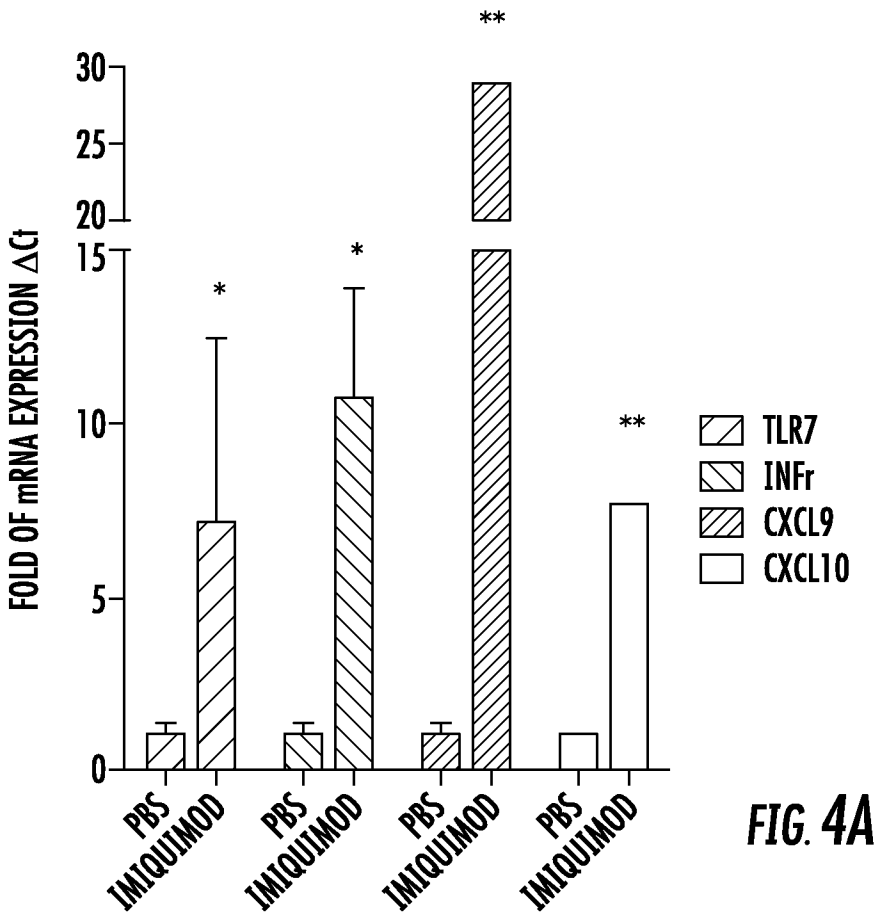


FIG. 4A

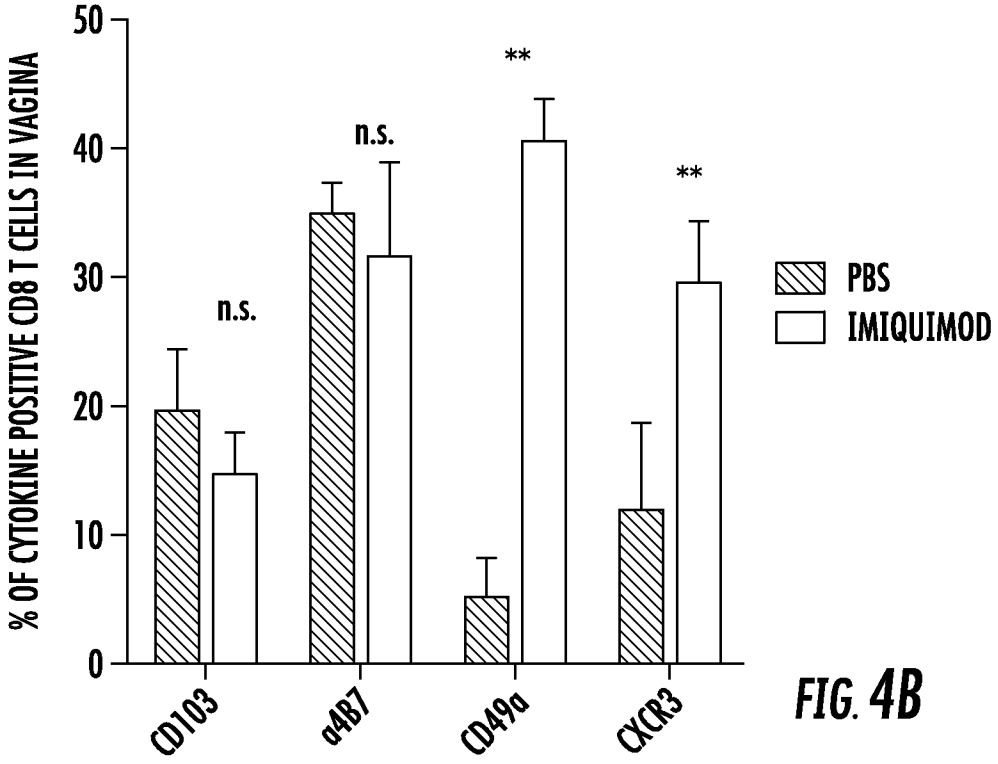

% OF CYTOKINE POSITIVE CD8 T CELLS IN VAGINA
50
40
30
20
10
0
n.s.
n.s.
**
**
PBS
IMIQUIMOD
CD103
α4B7
CD49a
CXCR3

FIG. 4B

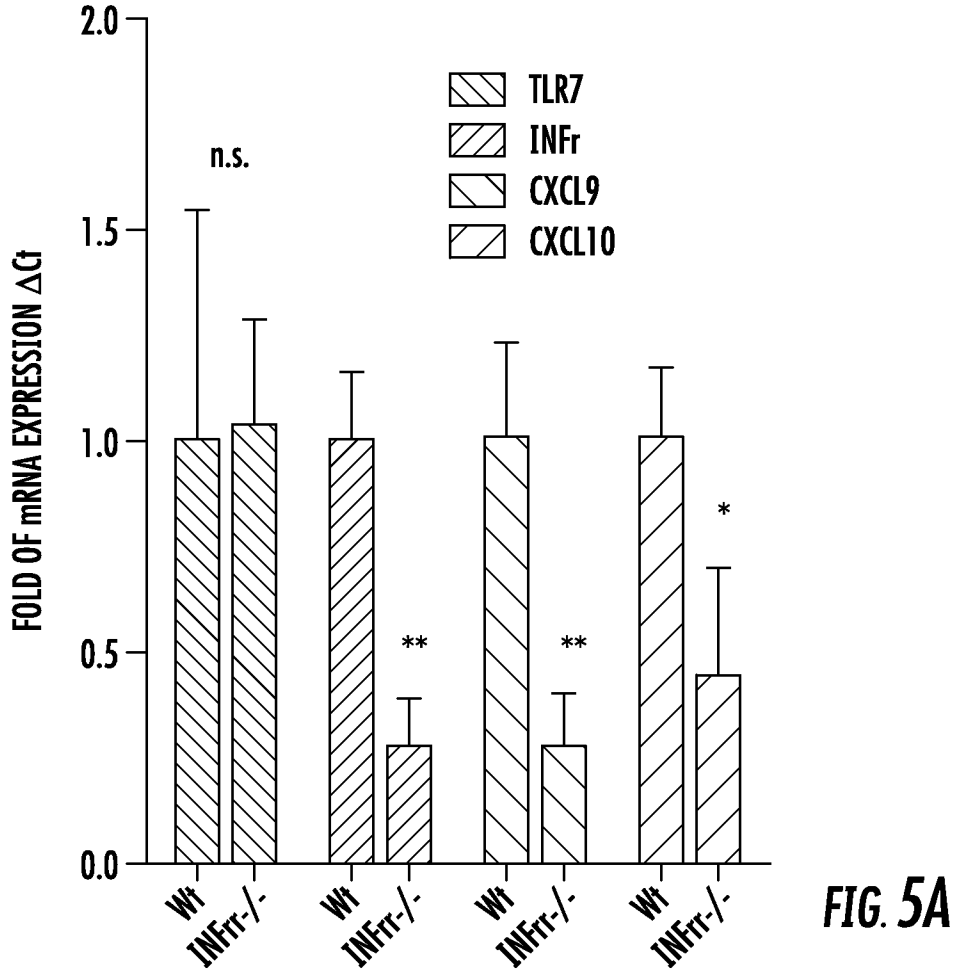

FOLD OF mRNA EXPRESSION ΔCt
2.0
1.5
1.0
0.5
0.0
n.s.
**
**
*
TLR7
INFr
CXCL9
CXCL10
Wt
INFrr-/-
Wt
INFrr-/-
Wt
INFrr-/-
Wt
INFrr-/-

FIG. 5A

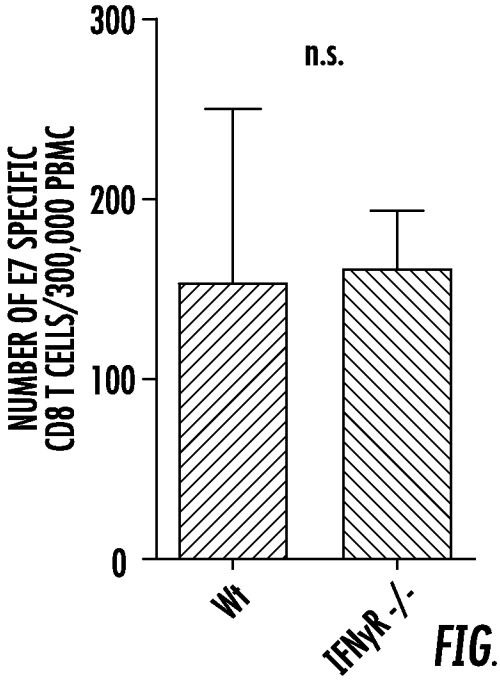


FIG. 5B

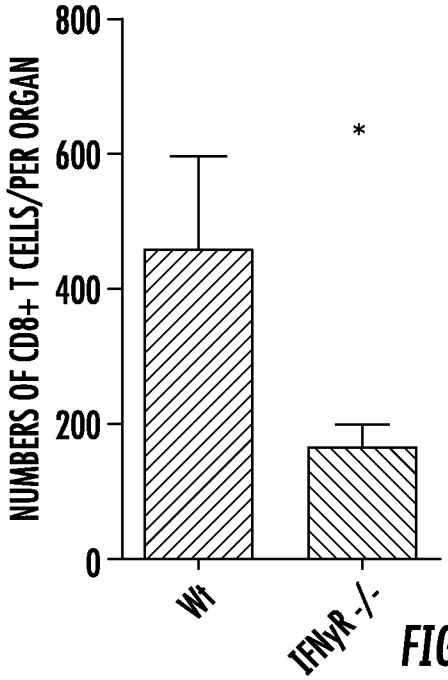


FIG. 5C

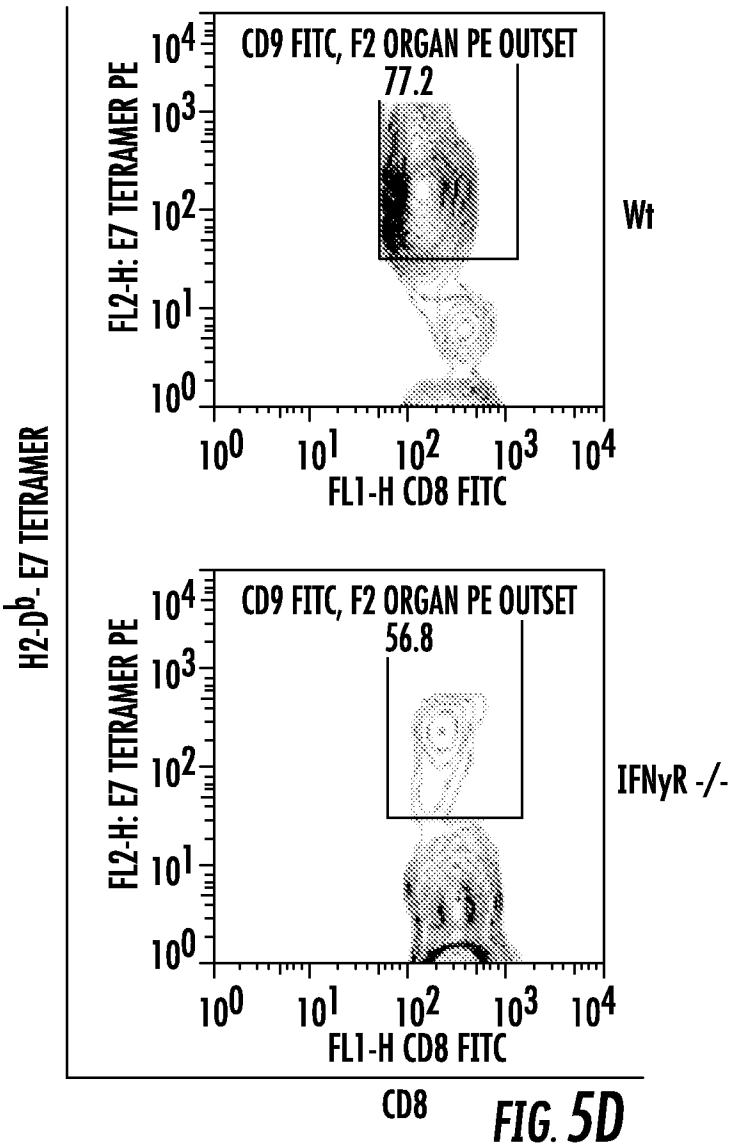


FIG. 5D

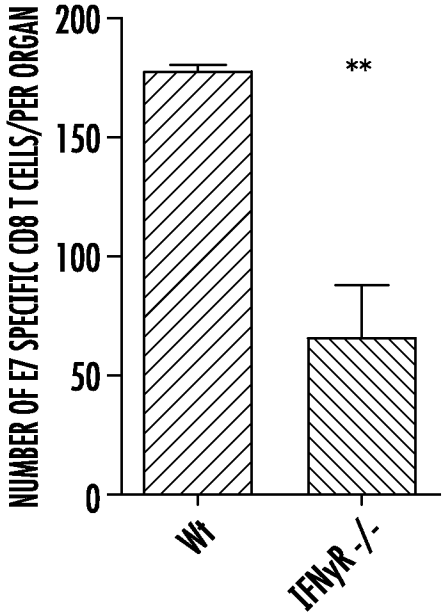


FIG. 5E

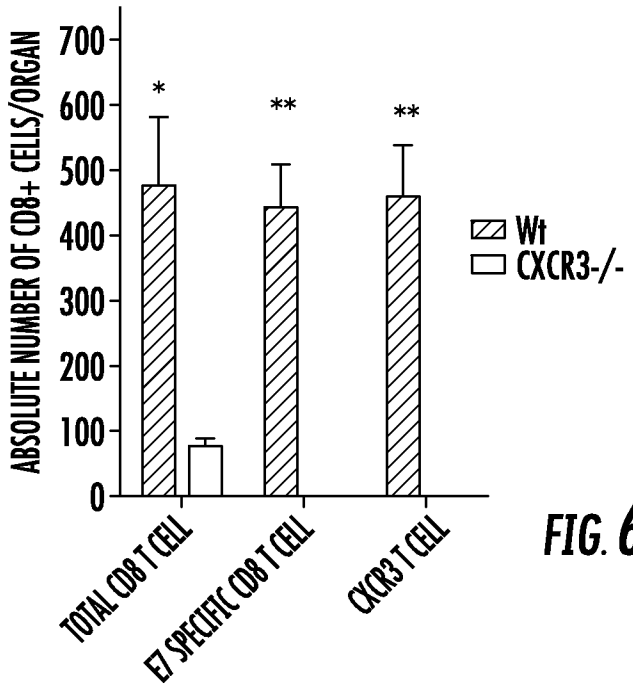

ABSOLUTE NUMBER OF CD8+ CELLS/ORGAN
700
600
500
400
300
200
100
0
*
**
**
Wt
CXCR3-/-
TOTAL CD8 T CELL
E7 SPECIFIC CD8 T CELL
CXCR3 T CELL

FIG. 6A

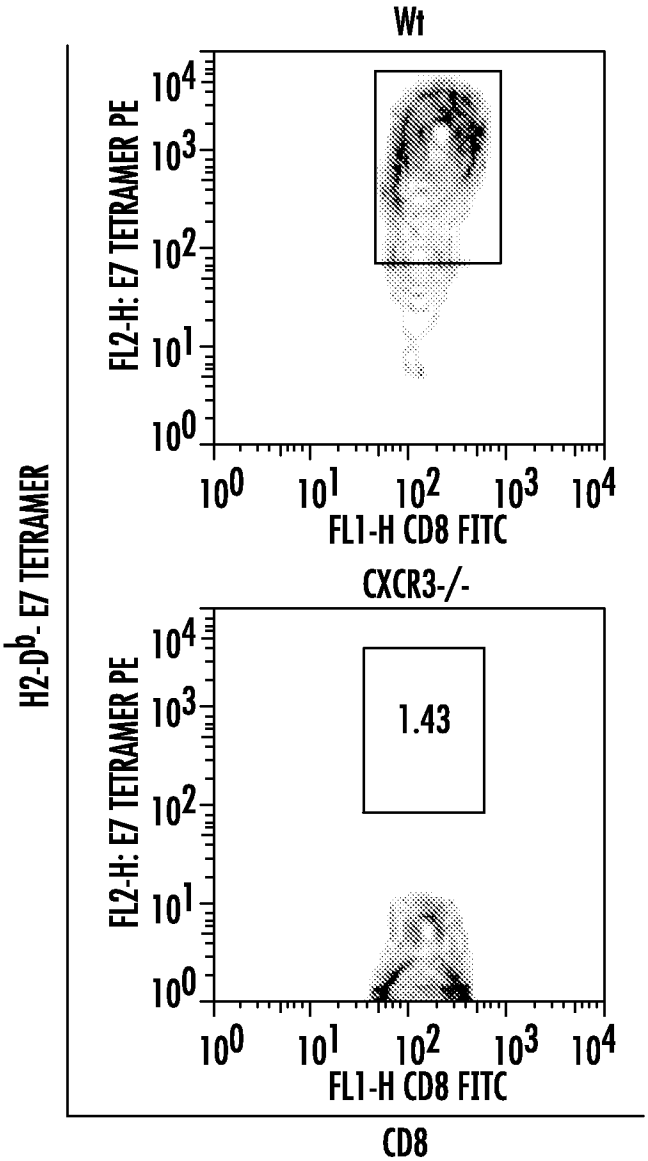

Wt
FL2-H: E7 TETRAMER PE
FL1-H CD8 FITC
CXCR3-/-
1.43
H2-Db- E7 TETRAMER
CD8

FIG. 6B

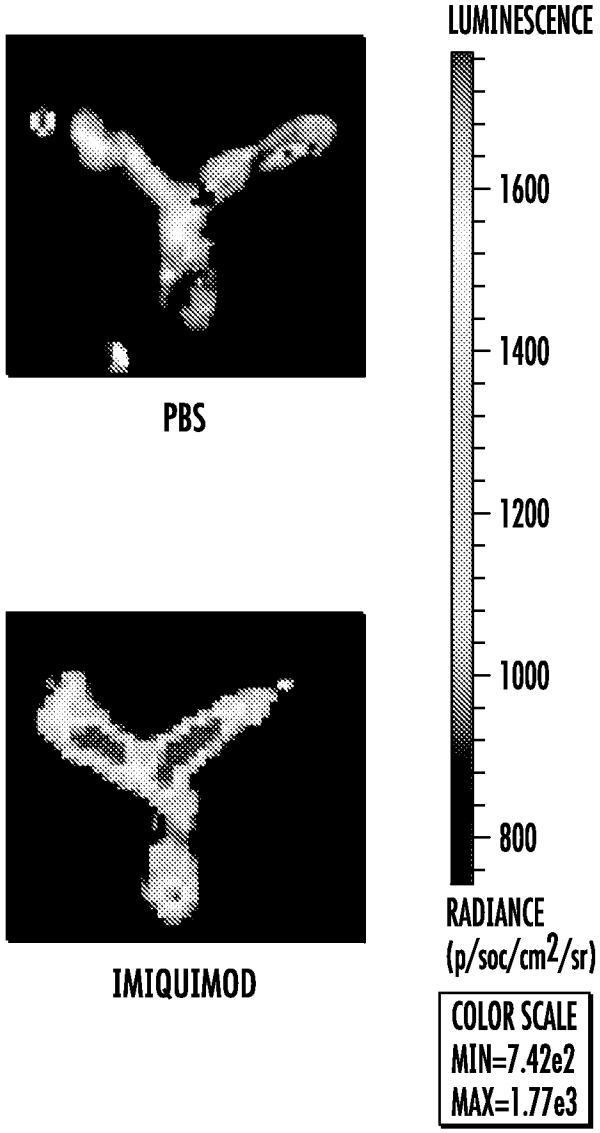

LUMINESCENCE
1600
1400
1200
1000
800
PBS
IMIQUIMOD
RADIANCE
(p/soc/cm2/sr)
COLOR SCALE
MIN=7.42e2
MAX=1.77e3

FIG. 7A

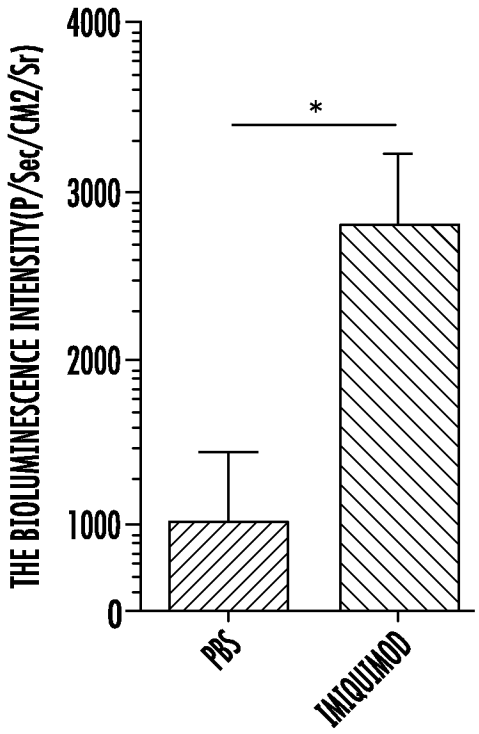

THE BIOLUMINESCENCE INTENSITY(P/Sec/(CM2/Sr)
4000
3000
2000
1000
0
*
PBS
IMIQUIMOD

FIG. 7B

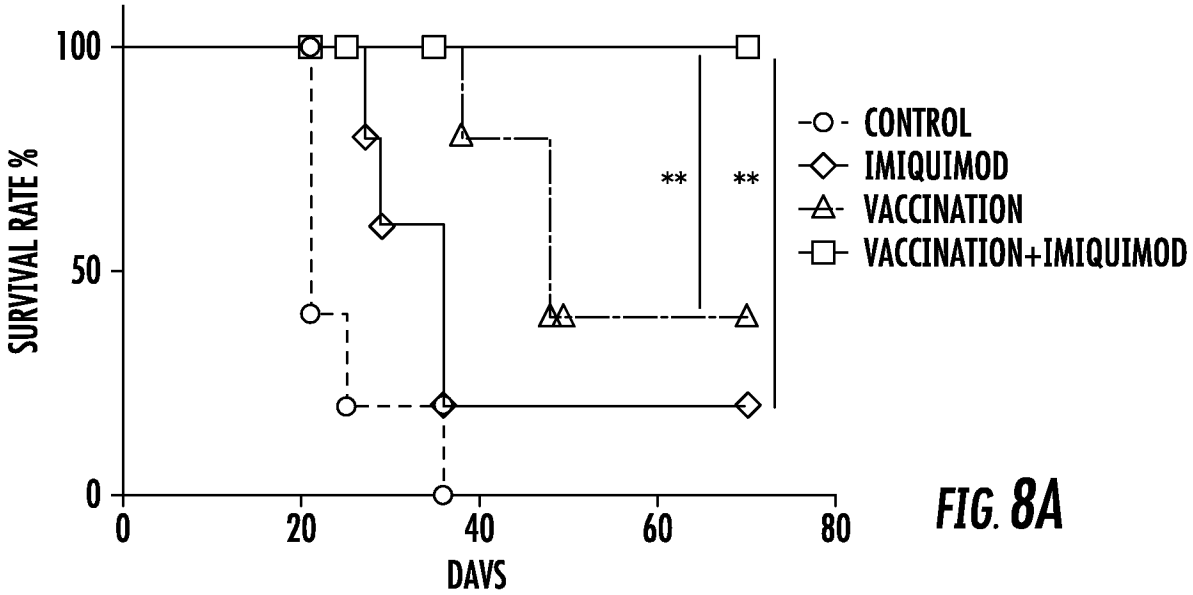


FIG. 8A

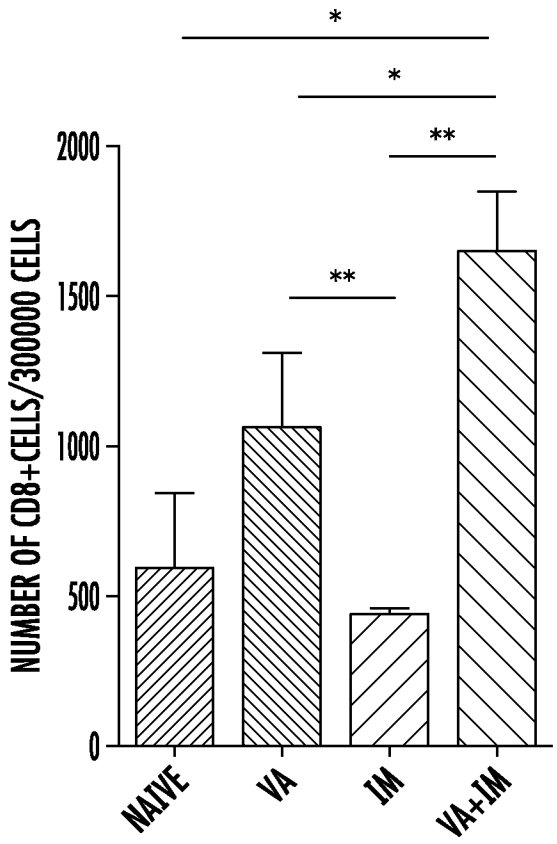


FIG. 8B

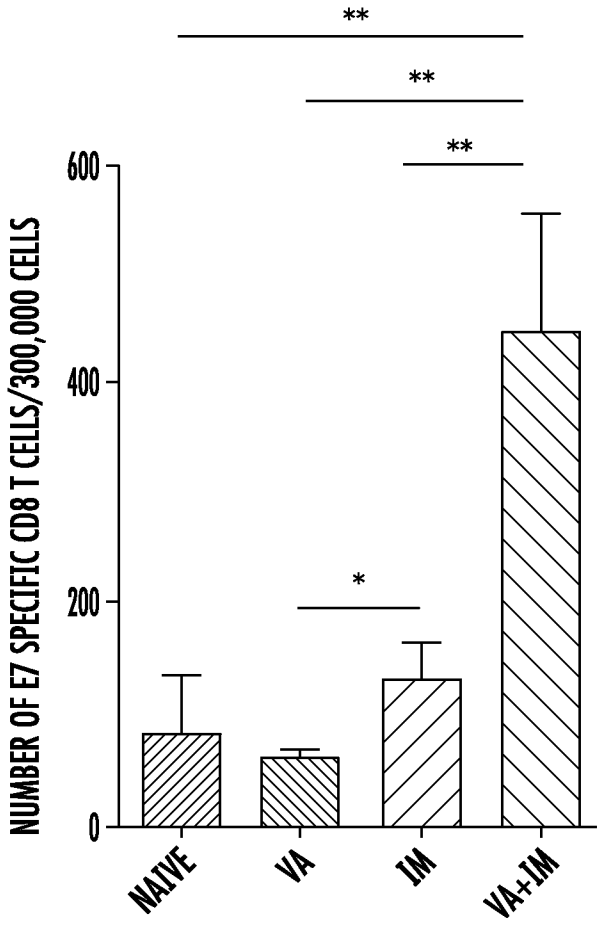


FIG. 8C

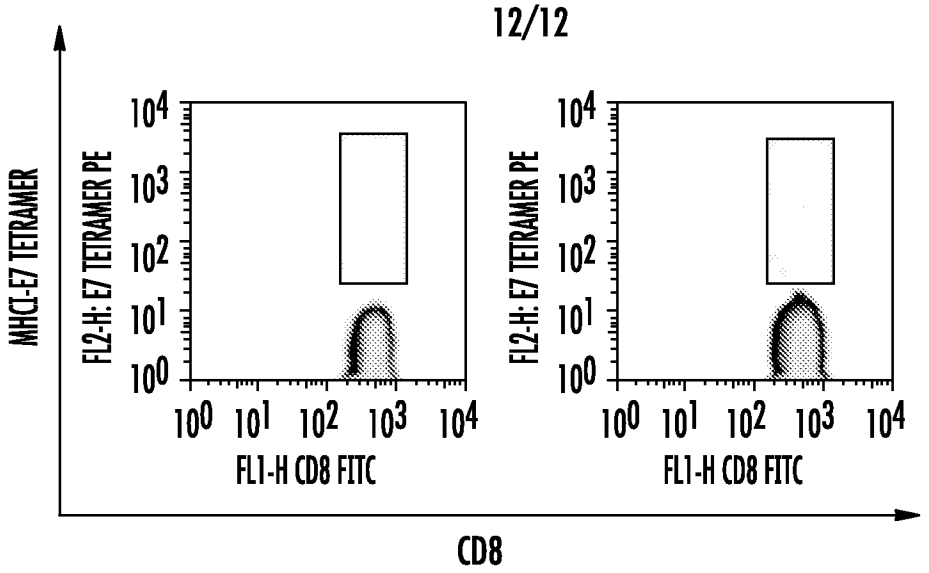


FIG. 9A

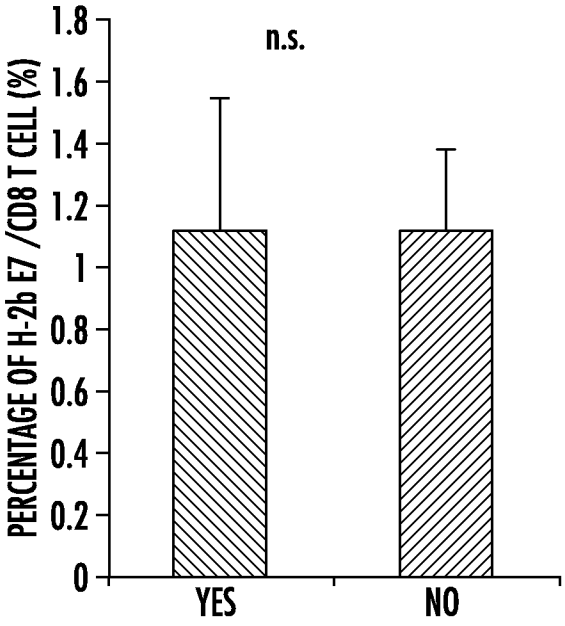


FIG. 9B

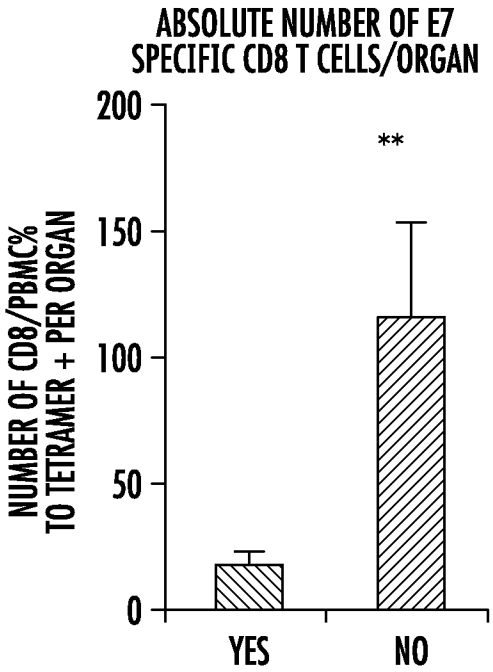


FIG. 9D

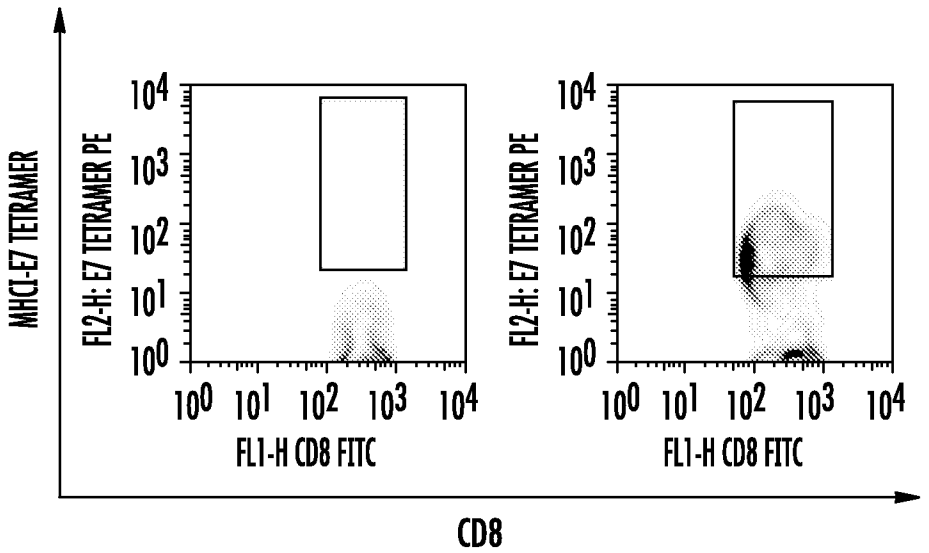


FIG. 9C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/023222

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/12(2006.01)i, A61K 39/39(2006.01)i, A61K 38/08(2006.01)i, A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 39/12; C12N 15/63; A61K 39/00; A61P 35/00; A61K 39/39; A61K 38/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:papillomavirus (HPV), vaccine, adjuvant, TLR agonist, CRT/E7 DNA, imiquimod

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DAAYANA, S. et al., "Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia", British Journal of Cancer, 16 March 2010 (Epub), Vol. 102, Pages 1129-1136 See abstract; pages 1130-1131, 1133.	1-45
X	CHUANG, CHI-MU et al., "Treatment with imiquimod enhances antitumor immunity induced by therapeutic HPV DNA vaccination", Journal of Biomedical Science, 28 April 2010, Vol. 17, Article No. 32, Internal pages 1-9 See abstract; internal pages 1-4, 7-8.	1-45
A	US 2010-0143408 A1 (BAUDIN, MARTINE et al.) 10 June 2010 See abstract; claims 50-70; paragraph [0120].	1-45
A	US 2012-0225090 A1 (WU, TZYY-CHOOU et al.) 6 September 2012 See abstract; claim 1; paragraphs [0122], [0164]-[0165].	1-45
A	US 2010-0330105 A1 (WU, TZYY-CHOOU et al.) 30 December 2010 See abstract; claims 1-20.	1-45

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 2015 (22.06.2015)	**29 June 2015 (29.06.2015)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
LEE, Jeong A
Telephone No. +82-42-481-8740

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2015/023222

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